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Flow Injection Atomic Absorption Assay Of Copper And Zinc In The Plasma Of Age Dependent Audiogenic Seizure Susceptible Mice

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FLOW INJECTION ATOMIC ABSORPTION ASSAY OF COPPER AND ZINC IN THE PLASMA OF AGE DEPENDENT AUDIOGENIC SEIZURE SUSCEPTIBLE MICE

by

Neal Dwain Byington

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Dated May 19, 1982

ABSTRACT OF DISSERTATION

by Neal Dwain Byington

FLOW INJECTION ATOMIC ABSORPTION ASSAY OF COPPER AND ZINC IN THE PLASMA OF AGE DEPENDENT AUDIOGENIC SEIZURE SUSCEPTIBLE MICE

Flow injection atomic absorption procedures with less than 10%RSD at 1.0 ppm were developed for the assay of copper and zinc in 10 µL samples of mouse plasma. The flow injection apparatus was designed with a pulseless liquid flow control system, regulated by inert gas pressure. The aspiration liquid is 6% t-butanol in water. After exhaustive parameter optimizations, copper assays were conducted with the conventional air-acetylene flame at 324.754 nm and zinc assays were conducted with the unconventional argon-hydrogen air diffusion flame at 213.856 nm. The argon-hydrogen flame system was chosen because it absorbs very little ultraviolet light.

The developed methods were used to determine the concentration of copper and zinc in plasma samples from six strains (C57BL/6J, LG/J, DBA/2J, SJL/J, BALB/cJ, LP/J) of male and female audiogenic seizure susceptible mice, aged 18 to 65 days. The results are presented in 24 sets of data and graphs of metal concentrations versus mouse age.

The copper concentration, within the precision of the data, is 1.2 ppm $\pm$ 0.2 SD for all sexes, strains, and ages. The zinc concentration, within the precision of the data, increases from 1.2 ppm $\pm$ 0.2 SD at twenty days of age to 1.8 ppm $\pm$ 0.2 SD at sixty days of age for all sexes and strains.

There is no correlation between the age dependence of the seizure susceptibility of any sex or strain of mice and the plasma concentrations or concentration ratios for either copper or zinc. At any age there is no correlation between the relative seizure susceptibility among the strains of mice and their concentrations or concentration ratios for either copper or zinc.

The results cast doubt on the significance of copper and zinc concentrations for the epilepsy of humans receiving anticonvulsant medication and on the validity of the use of audiogenic seizure susceptible mice as a model for epilepsy. Additional experiments are suggested.

DEDICATION

~~This dissertation is dedicated to my wife, Janice.~~ Her interest, support, encouragement, and presence were instrumental in making our leisurely progress through graduate school a generally pleasant and intellectually satisfying experience.

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I extend my thanks and gratitude to my research director, Professor Herschel Frye. His cheerful disposition, sharp wit, and vast breadth of interests enabled him to maintain an enjoyable, lively, and humane research group. His ability to judge when to leave a graduate student alone to develop, mature, and work at his own natural pace was priceless and unforgettable. I shall miss this very rare man and most sensitive mentor.

I extend my thanks and gratitude to the faculty and staff of the Department of Chemistry for their advice, patience, help, encouragement, and friendship during my tenure as a graduate student.

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Flow injection atomic absorption procedures with less than 10 %RSD at 1.0 ppm were developed for the assay of copper and zinc in 10 µL samples of mouse plasma. The flow injection apparatus was designed with a pulseless liquid flow control system, regulated by inert gas pressure. The aspiration liquid is 6% t-butanol in water. After exhaustive parameter optimizations, copper assays were conducted with the conventional air-acetylene flame at 324.754 nm and zinc assays were conducted with the unconventional argon-hydrogen air diffusion flame at 213.856 nm. The argon-hydrogen flame system was chosen because it absorbs very little ultraviolet light.

The developed methods were used to determine the concentration of copper and zinc in plasma samples from six strains(C57BL/6J, LG/J, DBA/2J, SJL/J, BALB/cJ, LP/J) of male and female audiogenic seizure susceptible mice, aged 18 to 65 days.

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CHAPTER ONE

1. Atomic Absorption Flow Injection Analysis

Any chromatographic system includes the injection of a sample into a continuously flowing stream of gas or liquid, separation of the sample into components as it is transported through the separation column by the flowing stream, and the transportation of the sample by the stream from the separation column to and through the detector. The continuously operating detector produces some form of output signal as a function of time as the sample passes through the detector.

There are, however, many analytical procedures that do not require separation of the sample into individual components. They only require detection of the injected sample by an analysis specific detector or the reaction of the injected sample with a reagent followed by detection with an analysis specific detector. This type of continuous flow analysis, with its origins in gas and liquid chromatography, was developed by Skeggs(1), and required the injection of sufficient sample to obtain a steady state signal and air segmentation between the injected samples. Most automated clinical analyzers operate on this basis.

Nagy, Feher, and Pungor later simplified this concept by eliminating the requirement for the injection of sufficient sample to obtain a steady state signal and the requirement for air segmentation between the injected samples(2). Their system injected the sample into a flowing stream of electrolyte, mixed the sample and electrolyte with a magnetic stirrer, and observed it with an electrochemical detector. They obtained sharp "gas chromatograph type" peaks.

This process was refined, patented, and popularized by Ruzicka and Hansen(3,4,5) and later by Stewart and Hare(6) who restricted the sample and solvent stream mixing to diffusion by the use of narrow bore tubing. These groups called the process "Flow Injection Analysis". In its most general form it includes the injection of a sample into a continuous stream of liquid or gas and the subsequent detection of that sample by a detector. The analysis design may also provide for the mixing of the sample or the reaction of the sample by any process between the points of injection and detection. The development of flow injection analysis, applicable diffusion mixing theory, applications, and instrumentation have been discussed by Betteridge(7) and Ranger(8).

1.1 Atomic Absorption Flow Injection Analysis

Wolf and Stewart extended the applications of flow injection analysis to atomic absorption spectroscopy(9). Their system included a depulsed positive displacement pump, an automatic four or six port rotary loop injection valve, an aqueous solvent system, small bore tubing, and an automatic integrator or analog recorder. They automatically injected 25 to 300 μL of sample into the nebulizer of an atomic absorption spectrometer.

Fukamachi and Ishibashi enhanced the sensitivity of atomic absorption flow injection analysis(10). They compared the sensitivities resulting from organic solvent streams with those from aqueous solvent streams. For 14 metals, sensitivity enhancements were reported for methanol, n-butyl acetate, and methyl iso-butyl ketone streams. The largest sensitivity enhancement was caused by n-butyl acetate.

This dissertation extends the sensitivity and application of atomic absorption flow injection analysis to small volumes of complex fluids such as plasma and serum. The sensitivity is enhanced by the use of mixed aqueous-organic solvent systems, systematic procedures for the selection of optimal instrument parameters, and air-acetylene and argon-hydrogen air diffusion flame systems.

The use of the mixed aqueous-organic solvent system, 6%(v/v) t-butanol in water, provides for enhanced sensitivity and does not cause precipitation of solutes from complex fluids such as serum and plasma. This work is applicable to the analysis of samples which are viscous, aqueous and/or organic, salty, and chemically complex.

The instrumental design is described in section 1.2. The experimental peak shapes are presented in Figure 1.1.1. The instrumental operating parameters and the experiments which led to their selection are discussed in section 1.3. In Table 1.1.1, minimal amounts of model compounds detectable by a very sensitive gas chromatographic method are shown for comparison of the sensitivity of this method to another very sensitive method(11). A referenced comparison of metal analysis parameters by various techniques is presented in Table 1.1.2.

The instrumental parameters for this version of atomic absorption flow injection analysis were developed to provide a means of analyzing very small volumes of mouse plasma for copper and zinc at the 1 ppm concentration level with a percent relative standard deviation(%RSD) of 10 percent or less. This was achieved. Minimal levels of 3 ng for zinc and 4 ng for copper at a signal to noise ratio of 2:1 are detectable. The major advantages of this system are the sensitivity, lack of matrix interferences, precision, and speed of analysis.

TABLE 1.1.1

THE MINIMAL AMOUNT OF MODEL COMPOUNDS DETECTABLE BY USE OF A SILICON DOPED HYDROGEN ATMOSPHERE FLAME IONIZATION DETECTOR

Model Compound	Minimal Detectable Amount, g (Signal to noise ratio, 2:1)
Aluminum hexafluoroacetylacetonate	6×10^{-13}
Ferrocene	2×10^{-12}
Chromium hexacarbonyl	2×10^{-11}
Tungsten hexacarbonyl	5×10^{-10}
Molybdenum hexacarbonyl	4×10^{-9}
Iron(III) trifluoroacetylacetonate	4×10^{-9}
Di-n-butyl sulfide	2×10^{-8}
Nitrobenzene	2×10^{-7}
Tetradecane	4×10^{-7}

Atomic Absorption Flow Injection Analysis Techniques

This Work

Copper	4×10^{-9}
Zinc	3×10^{-9}

TABLE 1.1.2

COMPARISON OF METAL ANALYSIS PARAMETERS BY VARIOUS TECHNIQUES

TECHNIQUE	DETECTION LIMITS		%RSD	SAMPLE SIZE
	Cu	Zn		MINIMUM, μL
AA	0.15 ppm	0.025 ppm	1-2	1000
AA-GF	0.6 pg	0.3 pg	5-200	1-300
ICP	0.005 ppm	0.004 ppm	1-2	1000
DCP	0.002 ppm	0.003 ppm	8-10	1000
FIA-ICP	Mn (10 ng)		2	25
FIA-AA	4 ng	3 ng	2	25-300
FIA-AA	5 ng	---	5	50
FIA-AA	4 ng	3 ng	2-10	1-10

TABLE 1.1.2 (CONTINUED)

COMPARISON OF METAL ANALYSIS PARAMETERS BY VARIOUS TECHNIQUES

TECHNIQUE	SOLVENT	SAMPLE INTRODUCTION	REFERENCE
AA	Aqueous/Organic	Aspiration	12
AA-GF	Aqueous/Organic	Syringe	13
ICP	Aqueous/Organic	Aspiration	14
DCP	Aqueous/Organic	Aspiration	15
FIA-ICP	Aqueous	Loop Injector	16
FIA-AA	Aqueous	Loop Injector	9
FIA-AA	n-butyl acetate	Syringe	10
FIA-AA	6%(v/v) t-butanol in water	Syringe	This Work

TABLE 1.1.2 (CONTINUED)

COMPARISON OF METAL ANALYSIS PARAMETERS BY VARIOUS TECHNIQUES

AA	Atomic Absorption Spectroscopy
FIA	Flow Injection Analysis
ICP	Inductively Coupled Plasma Emission Spectroscopy
DCP	Direct Current Plasma Emission Spectroscopy
GF	Graphite Furnace
ppm	Part Per Million
pg	Picogram
ng	Nanogram
µL	Microliter

FIGURE 1.1.1

Experimental Peak Shapes

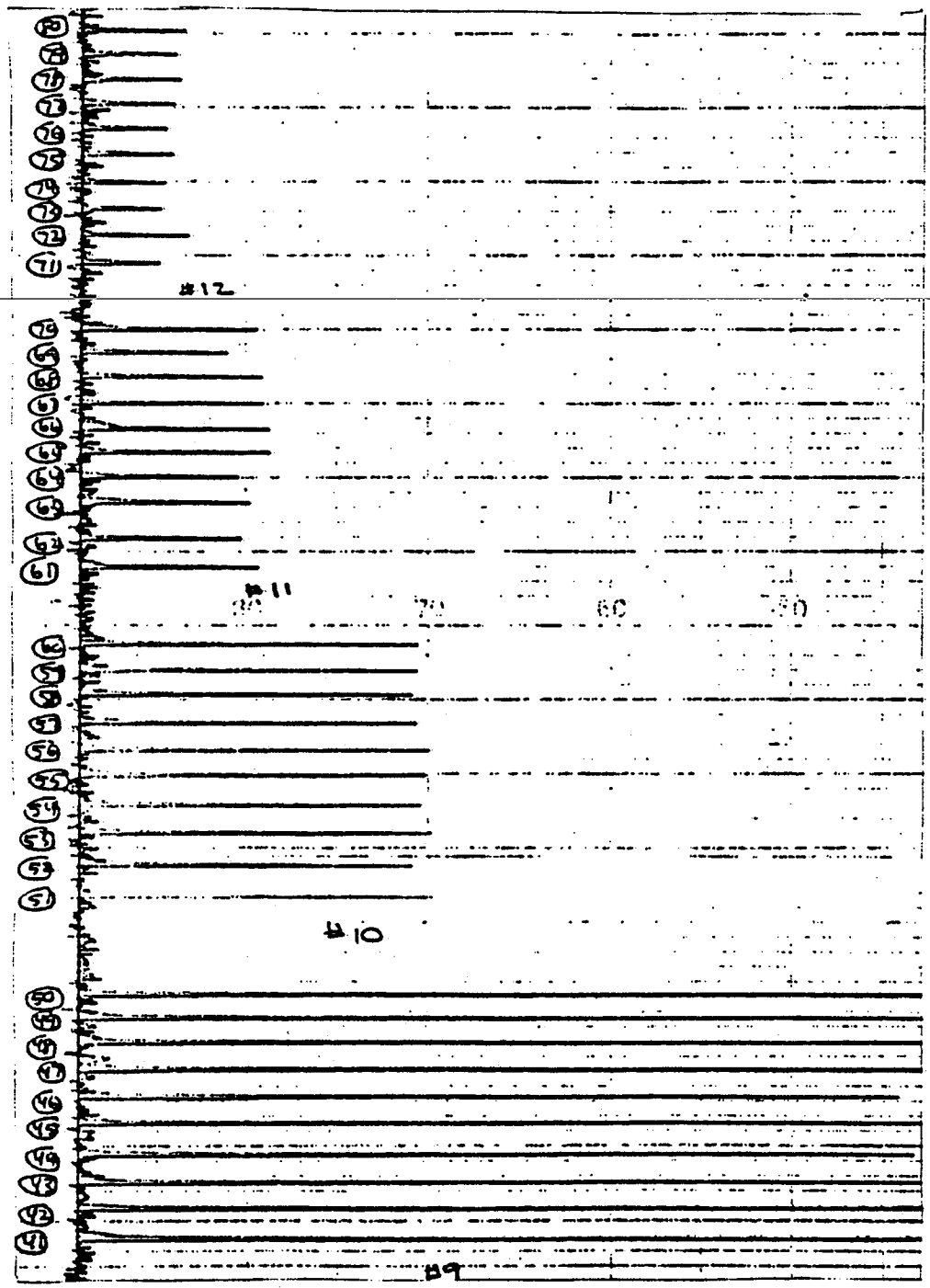
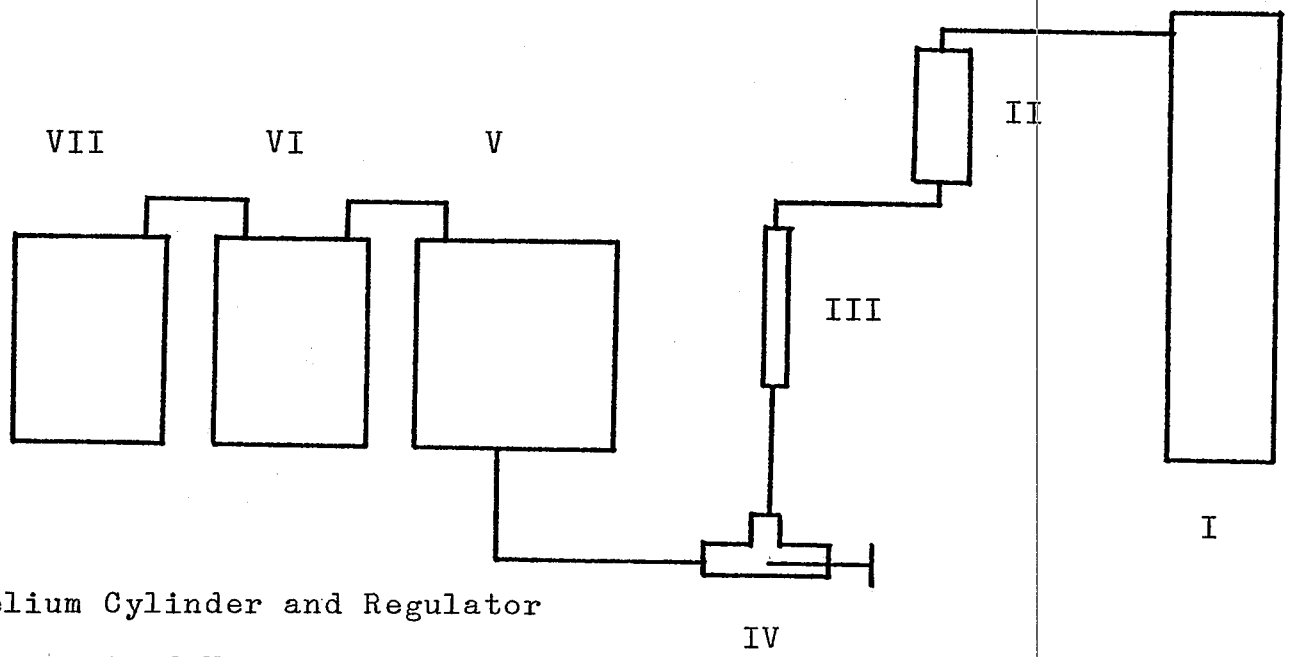

#12
#11
#10
#9

FIGURE 1.2.1

Flow Injection Apparatus



I Helium Cylinder and Regulator

II Pressurized Vessel for Solvent

III Flow Restriction Column

IV Syringe Injection Port

V Atomic Absorption Spectrometer

VI Recorder Readout Accessory

VII Analog Stripchart Recorder

1.2 Instrument Design for Flow Injection Analysis

A block diagram of the apparatus is given in Figure 1.2.1. The flow injection apparatus consists of a helium cylinder and regulator(I) which is connected to a 5 liter Millipore pressurized vessel(II) which is connected to a flow restriction column(III) which is connected to a 1/16" Swagelok stainless steel tee injection port(IV) which is connected to the nebulizer on the Perkin-Elmer Model 303 atomic absorption spectrometer(V). The atomic absorption signal from the spectrometer is sent to the recorder readout accessory(VI) and then to a Linear analog stripchart recorder(VII).

The 5 liter Millipore pressure vessel(II) functions as a pulseless solvent pump. It is pressurized by helium from a conventional tank and regulator(I). The vessel is equipped with a pressure gauge and a valve at the solvent outlet.

The flow restriction column(III) is a length of 6 mm O. D. x 16.7 cm soft glass tube filled with Corning Controlled Pore Glass, CPG-10-500, with 120/200 mesh. The ends are 1/4" to 1/16" end drilled stainless steel Swagelok reducing unions with 2.0 micron pressed stainless steel filters. The ferrule is an Alltech 6 mm to 1/4" teflon reducing ferrule.

The injection port(IV) is a 1/16" stainless steel Swagelok tee with a septum at one end of the tee. The septum is cut from a regular blue Alltech septum with a #00 stopcock borer and trimmed to a cone shape with a single edged razor blade. Samples are injected with a 10 uL Hamilton #701 syringe.

The tubing which connects the pressure vessel to the flow restriction column, the flow restriction column to the injection port, and the injection port to the nebulizer is 1/16" O. D. x 0.023" I. D. teflon tubing from Laboratory Data Control. All connections are done with 1/16" stainless steel Swagelok fittings.

1.3 Optimal Parameter Development for the Instrument

1.3.1 Analytical Wavelengths

The choice of the analytical wavelengths of 324.754 nm for copper and 213.856 nm for zinc was made by an analysis of the various copper and zinc lines with respect to their energy levels, statistical weights of the upper levels(g), Einstein transition probabilities(A), Ladenburg oscillator strengths(f), and possibly interfering lines from other elements(17). The intent was to maximize the signal strength by the choice of ground state lines with large gA and gf values and to have all possible interfering lines in the vicinity of the chosen lines be either from elements of very low concentration in the sample such as the rare earth elements or be non-ground state lines with low gA and gf values. These goals were accomplished by a search of published experimental transition probabilities.

Table 1.3.1.1 is a listing of the energy levels for possibly interfering lines which lie within 0.65 nm of the copper line at 324.754 nm. Table 1.3.1.2 is a listing of the energy levels for possibly interfering lines which lie within 1.95 nm of the zinc line at 213.856 nm.

The optical slit width of the spectrometer is 0.65 nm for assays of copper at 324.754 nm and 1.95 nm for assays of zinc at 213.856 nm(12). Table 1.3.1.3 is a listing of possible other element spectral overlap lines which lie in close proximity to the copper line at 324.754 nm and the zinc line at 213.856 nm. Lines which cause spectral overlap generally lie within 0.1 nm of the analytical line.

A series of absorbance tests for metal interference was conducted to determine if other metal solutions would absorb at the copper or zinc wavelengths. The test solutions were 1000 ppm atomic absorption standards. Table 1.3.1.4 lists the metals tested and their absorbance at the analytical wavelengths for copper and zinc of 324.754 nm and 213.856 nm respectively.

A small negative absorbance from chromium and a small positive absorbance of similar magnitude from nickel were observed at the copper wavelength. The magnitude of the absorbance is equivalent to a copper concentration of approximately 0.25 ppm. This is considered insignificant when compared to the 1000 ppm standard. These small absorbancies are of unknown origin but may be due to impurities in the standard solutions. None of the other 1000 ppm metal solutions tested produced any detectable absorbance at either the copper or zinc wavelengths

TABLE 1.3.1.1

Ground State Lines Near the Copper Line at 324.754 nm for Various Metals

324.104 - 324.754 - 325.404

Wavelength nm	Metal	Energy Level ^{o}K	gA 10^{8}/sec	gf	log gf
324.754	Cu I	0 - 30784	4.1	0.64	-0.19
324.140	Eu I	0 - 30842	0.082	0.13	-0.89
324.603	Eu I	0 - 30798	0.40	0.06	-1.19
324.755	Eu I	0 - 30784	0.89	0.14	-0.85
325.037	Sm II	0 - 30757	0.94	0.15	-0.83
324.446	Th I	0 - 30813	0.56	0.09	-1.06
324.987	Th I	0 - 30762	0.48	0.08	-1.12
324.199	Ti II	0 - 30837	5.8	0.91	-0.04
324.153	Tm II	0 - 30841	1.1	0.17	-0.76
325.805	Tm II	0 - 30684	0.87	0.14	-0.86

TABLE 1.3.1.2

Ground State Lines Near the Zinc Line at 213.856 nm for Various Metals

211.906 - 213.856 - 215.806

Wavelength nm	Metal	Energy Level ^{o}K	gA 10^{8}/sec	gf	log gf
213.856	Zn I	0 - 46745	19	1.3	0.12
214.438	Cd II	0 - 46619	106	7.3	0.86
211.954	Ir I	0 - 47165	0.74	0.05	-1.30
212.794	Ir I	0 - 46979	2.1	0.14	-0.84
215.581	Ir I	0 - 46372	1.5	0.11	-0.97
215.784	Os I	0 - 46328	1.1	0.08	-1.10
214.423	Pt I	0 - 46622	1.8	0.13	-0.90
215.667	Re I	0 - 46353	1.2	0.08	-1.80
212.739	Sb I	0 - 46991	0.43	0.03	-1.53
214.275	Te I	0 - 46653	5.8	0.40	-0.40
212.672	Yb II	0 - 47006	0.41	0.03	-1.56

TABLE 1.3.1.3

Metal Lines Which Can Overlap the Copper Line at 324.754 nm and the Zinc Line at 213.856 nm

Wavelength nm	Metal	Energy Level ^{o}K	gA 10^{8}/sec	gf	log gf
324.754	Cu I	0 - 30784	4.1	0.64	-0.19
324.755	Eu I	0 - 30784	0.89	0.14	-0.85
324.766	Hf I	2375 - 33139	0.67	0.11	-0.98
324.747	Nb II	7901 - 38685	2.9	0.46	-0.33
324.746	Tm II	8770 - 39554	2.3	0.37	-0.43
213.856	Zn I	0 - 46745	19	1.3	0.12
213.853	Cu I	11203 - 57949	0.51	0.04	-1.46

TABLE 1.3.1.4

Interference Absorbance by Various Metals at Copper and Zinc Wavelengths

Metal, 1000 ppm	Cu, Absorbance at 324.754 nm	Zn, Absorbance at 213.856 nm
Na	0.0000	0.0000
K	0.0000	0.0000
Li	0.0000	0.0000
Ca	0.0000	0.0000
Mg	0.0000	0.0033
Cr	-0.0032	0.0000
Ni	0.0033	0.0000
Fe	0.0000	0.0000
Mn	0.0000	0.0000
Cu	---	0.0000
Zn	0.0000	---

1.3.2 Optical System

TABLE 1.3.2.1

Optical System for the Perkin-Elmer Model 303 Atomic Absorption Spectrometer

Grating	Czerny-Turner
Dispersion	UV 6.5 Å/mm
Dispersion	VIS 13.0 Å/mm
Focal Length	400 mm
UV Grating	2880 lines/mm
VIS Grating	1440 lines/mm
UV Blazed	2100 Å
VIS Blazed	6000 Å
Slit Openings	0.03 mm
	0.10 mm
	0.30 mm
	1.00 mm
	3.00 mm
	10.00 mm
UV Spectral Band Width	0.2 Å
VIS Spectral Band Width	0.4 Å

TABLE 1.3.2.2

Optical Slit Dimensions for the Perkin-Elmer Model 303 Atomic Absorption Spectrometer

Slit	mm	UV, Å	VIS, Å	UV, nm	VIS, nm
1	0.03	0.195	0.39	0.0195	0.039
2	0.10	0.65	1.3	0.065	0.13
3	0.30	1.95	3.9	0.195	0.39
4	1.0	6.5	13	0.65	1.3
5	3.0	19.5	39	1.95	3.9
6	10.0	65	130	6.5	13.0

1.3.3 Flame Composition

The conventional air-acetylene flame was chosen for the atomic absorption assay of copper. This flame is ideal for the analysis of copper since the flame does not have any significant absorbance at the convenient analytical wavelength of 324.754 nm. The 2500°C temperature of this flame is adequate for over 98 percent atomization of the copper(18). This atomization process for copper is not hindered by flame assisted compound formation, ionization, or wide variations in either the sample pH or the fuel-air ratio.

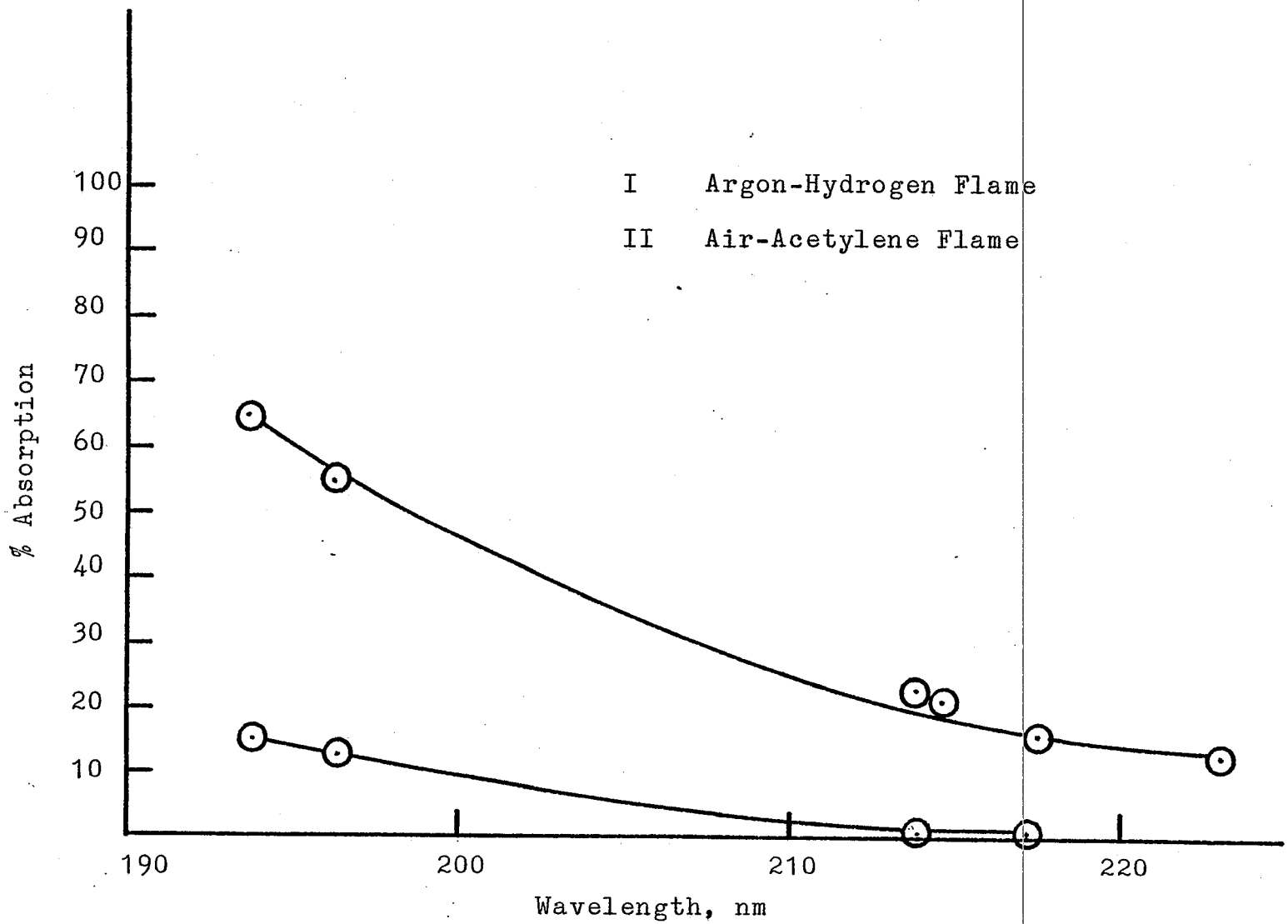
The nonconventional argon-hydrogen flame was chosen for the zinc atomic absorption analysis(19). In this system the argon replaces both the nebulizer air and the make-up air in the premix burner. The actual combustion occurs between the hydrogen and the air which surrounds the exterior of the burner head. There is no premixing of fuel and oxidant inside the premix burner chamber.

The major advantage of the argon-hydrogen flame is the negligible 0.6 percent absorption at the analytical wavelength of 213.856 nm compared to the approximately 21 percent signal absorption at this wavelength by the air-acetylene flame(12).

Figure 1.3.3.1 is a plot of the percentage absorption by the flame versus wavelength for the air-acetylene and argon-hydrogen flames. Greater precision and sensitivity are additional advantages obtained by the use of this flame system rather than the air-acetylene flame in zinc assays. This may in part be due to a lower flame temperature for the argon-hydrogen flame. The results of the experiments in which potassium was added to cesium solutions imply that the argon-hydrogen flame temperature is considerably lower than the air-acetylene flame temperature since cesium is not ionized in this flame whereas in the air-acetylene flame it is 90 percent ionized(19).

FIGURE 1.3.3.1

Percent Absorption versus Wavelength for the Two Flames


I Argon-Hydrogen Flame
II Air-Acetylene Flame
% Absorption
100
90
80
70
60
50
40
30
20
10
190
200
210
220
Wavelength, nm

1.3.4 Burner Head and Nebulizer Selection

A set of one ppm copper solutions was analyzed with single and triple slot burner heads and with old and new Perkin-Elmer nebulizers. Optimal results were obtained by the use of a triple slot burner head with the new nebulizer as indicated in Table 1.3.4.1. This arrangement was used in our analytical work.

TABLE 1.3.4.1

Burner Head and Nebulizer Comparison

Absorbance	%RSD	n	Burner Head	Nebulizer
0.019	3.2	10	Triple Slot	New
0.012	7.7	10	Triple Slot	Old
0.014	9.5	10	Single Slot	New
0.010	19.9	10	Single Slot	Old

1.3.5 Burner Head Height Selection

The burner height for atomic absorption is the distance in cm that the top of the burner head lies below the bottom of the light path of the hollow cathode lamp. The burner height with the largest burner height parameter(BHP) is the optimal burner height. A BHP is defined for a specified burner height as the product of the absorbance times the inverse of the %RSD for a set of data obtained under otherwise identical analytical conditions.

Tables 1.3.5.1 and 1.3.5.2 give data for copper and zinc, respectively. The information in these tables provided the basis for the selection of a burner height of 1.5 cm for copper and 4.0 cm for zinc.

TABLE 1.3.5.1

Selection of the Burner Height for Copper Standard(2 ppm)

Burner Height, cm	Absorbance	%RSD	BHP	n
1.0	0.067	3.0	0.022	10
1.5	0.065	2.7	0.024	10
2.0	0.062	3.1	0.020	10
2.5	0.063	3.7	0.017	10
3.0	0.061	3.6	0.017	10
3.5	0.060	4.9	0.012	10
4.0	0.057	3.5	0.016	10
4.5	0.057	4.3	0.013	10
5.0	0.055	5.1	0.011	10

TABLE 1.3.5.2

Selection of the Burner Height for Zinc Standard(2 ppm)

Burner Height, cm	Absorbance	%RSD	BHP	n
1.0	0.056	3.7	0.015	10
1.5	0.070	6.8	0.010	10
2.0	0.073	3.6	0.020	20
2.5	0.078	6.1	0.013	10
3.0	0.081	4.5	0.018	10
3.5	0.081	4.7	0.017	10
4.0	0.082	3.4	0.024	10
4.5	0.077	4.4	0.018	10
5.0	0.073	7.4	0.010	10

1.3.6 Selection of the Fuel/Oxidant Ratio

Two significant variables which are influenced by the fuel/oxidant ratio are the availability of electrons in the flame and the temperature of the flame. Electrons must be available to combine with gaseous metal ions to produce neutral metal atoms. Their availability is increased in fuel rich flames. The temperature of the flame influences the density of free neutral atoms at the various electronic levels, the flame velocity, and the ability of the flame to form and decompose various compounds in the flame.

The selection of an optimal fuel/oxidant ratio is actually a balancing of all variables to obtain a maximal %RSD. The optimal ratio is that ratio which results in a maximal value for the product of the absorbance times the inverse of the %RSD for a series of assays where only the fuel/oxidant ratio is varied. Table 1.3.6.1 indicates that the optimal ratio for copper is obtained when the flowmeter scale in arbitrary units is 9.5 for acetylene and 9.0 for air.

The fuel/oxidant ratio was not determined for the argon-hydrogen flame. In this system the oxidant is the oxygen in the air that diffuses into the flame area from the atmosphere that surrounds the burner head.

Settings for the flowmeter scale, in arbitrary units, of 9.0 for argon and 5.0 for hydrogen were used for all zinc assays.

TABLE 1.3.6.1

Selection of the Fuel/Oxidant Ratio for Copper Standard(5 ppm)

Flowmeter Scale in Arbitrary Units					
Acetylene	Air	Absorbance	%RSD	Absorbance/%RSD	n
9.0	9.0	0.113	2.8	0.040	10
9.5	9.0	0.113	2.0	0.057	10
10.0	9.0	0.114	3.2	0.036	10
10.5	9.0	0.109	5.9	0.018	10

1.3.7 Sample Injection Size

The selection of an optimal sample injection volume requires compromises to be made with respect to an adequate absorbance value, a minimal %RSD, a sufficient volume of mouse plasma, and the number of required assays.

The sample volume of blood available from the various strains of mice without harm to the mice is in the range of 50 to 200 µL. This will provide a plasma volume of 25 to 100 µL. That must be a sufficient volume for at least one assay for both copper and zinc. The available sample volume limits the injection size to a maximum of 25 µL.

The optimal injection volume is that volume which results in a maximal value for the product of the absorbance times the inverse of the %RSD for a series of assays where only the injection volume is varied. Two series of assays were conducted with 2 ppm standard solutions of copper and zinc where only the injection volume was varied over the range of 1 to 10 µL. A separate series was conducted with a 1 ppm copper standard where only the injection volume was varied between 10 and 25 µL.

These results are listed in Table 1.3.7.3. An analysis of the combined results from the three tables suggests an injection volume of 10 µL, as a compromise.

TABLE 1.3.7.1

Sample Injection Sizes for Copper Standard(2 ppm)

Volume, µL	Absorbance	%RSD	n	(Absorbance/%RSD)/µL
10	0.025	3.1	10	0.00081
9	0.022	6.2	10	0.00039
8	0.020	3.6	10	0.00069
7	0.018	5.1	10	0.00050
6	0.016	4.8	10	0.00056
5	0.013	6.3	10	0.00041
4	0.011	6.5	10	0.00042
3	0.0080	10.7	10	0.00025
2	0.0053	15.4	10	0.00017
1	0.0020	27.6	10	0.00007

TABLE 1.3.7.2

Sample Injection Sizes for Zinc Standard(2 ppm)

Volume, µL	Absorbance	%RSD	n	(Absorbance/%RSD)/µL
10	0.036	9.7	10	0.00037
9	0.035	12.2	10	0.00032
8	0.031	7.6	10	0.00051
7	0.027	10.3	10	0.00037
6	0.025	12.4	10	0.00034
5	0.021	9.8	10	0.00043
4	0.017	15.5	10	0.00027
3	0.013	14.0	10	0.00031
2	0.0082	15.4	10	0.00027
1	0.0040	27.1	10	0.00015

TABLE 1.3.7.3

Large Sample Injection Sizes for Copper Standard(1 ppm)

Volume, µL	Absorbance	%RSD	n	(Absorbance/%RSD)/µL
25	0.048	12.5	20	0.00015
10	0.019	8.1	20	0.00023

1.3.8 Solvent Composition

Several different mixtures of 6% organic solvents with water were studied as aspiration solvents. The basis for these studies was literature reports of sensitivity enhancement by the use of organic solvent systems in atomic absorption spectroscopy(20). Some systems that used acetone reported three- to four-fold increases in sensitivity.

The sensitivity enhancement is due to modification of solution parameters which result in the alteration of several different nebulizer and flame processes. The mixed organic-aqueous solvents, when compared to water, generally have a lower viscosity and surface tension. These trends are similar to those found when organic solvents are compared with water. The advantages of mixed solvents are thus similar to the advantages of organic solvents but of a lesser magnitude. A solution of lower viscosity and surface tension produces, via a nebulizer, an aerosol with a smaller and more uniform droplet size. As a result, a larger percentage of aerosol enters the flame and is thus available for detection. The increased surface to volume ratio in the smaller droplets results in the more rapid evaporation of solvent from a solvent aerosol solution of metal ions.

This effect is desirable because it provides additional residence time for the ion to absorb heat from the flame. The ion thus attains a higher effective temperature which ultimately will result in a larger percentage of neutral metal atoms in the flame. The organic solvent generally has a lower heat of vaporization. The requirement for less heat from the flame to vaporize the solvent has the desirable effect of increasing the flame temperature. It also has the undesirable effect of decreasing the cooling of the burner head and increasing the flame propagation velocity. These effects increase the likelihood of a flashback.

The atomic absorption nebulizer can only nebulize solutions. Therefore, the mixed solvent system must not cause precipitation of the mouse plasma sample. This requirement eliminates the use of pure organic solvents and any mixed organic-aqueous solutions with a high organic solvent concentration.

Ketones, esters, and alcohols have been reported to yield stable flames. Based upon the reported use of 6% n-butanol in water(21), the limited single phase solubility of many organic solvents in water, and the plasma sample solubility requirements, a series of eight aqueous nebulizer solutions containing 6% organic solvent in water was tested.

The results are presented in Table 1.3.8.1. The solvent of choice was 6% t-butanol in water.

TABLE 1.3.8.1

Solution Effects on Flame Copper Absorbance

Solution, 6% in Water	Copper, Absorbance(%RSD) 5 ppm	2 ppm	1 ppm	0.5 ppm
n-propanol	---	0.0022(7.0)	0.0012(11)	0.00045(14)
iso-propanol	---	0.011(18)	0.0045(19)	0.008(24)
allyl alcohol	Strong absorbance with a noisy flame			
ethyl acetate	Strong absorbance with a noisy flame			
n-butanol	0.017(5.6)	0.068(11)	0.025(23)	---
iso-butanol	---	0.034(11)	0.015(21)	0.005(25)
sec-butanol	Strong absorbance with a noisy flame			
t-butanol	---	0.078(8.8)	0.033(14)	0.014(13)
water(100%)	---	0.046(8.5)	0.019(18)	0.008(29)

1.3.9 Solvent Flow

The rate with which the injected sample and the 6%(v/v) t-butanol solvent are pumped into the nebulizer determines the amount of sample in the light path and thus the magnitude of the absorbance signal and the precision of that signal. The flow rate of 6% t-butanol into the nebulizer is set by adjusting the helium pressure on the solvent pressure reservoir. A calibration curve of helium reservoir pressure versus flow rate is presented in Table 1.3.9.1 and Figure 1.3.9.1.

In order to determine the optimal flow rate for the analysis of copper and zinc, a set of 5 ppm zinc standards was analyzed under fixed experimental conditions at various flow rates. The zinc absorbances at the various flow rates were determined at 213.856 nm. The results of this experiment are listed in Table 1.3.9.2. The optimal flow rate of 6% t-butanol has a maximal value of the product of the absorbance times the inverse of the %RSD. Figures 1.3.9.2 and 1.3.9.3 are graphs of the zinc absorbance versus flow rate and the %RSD versus flow rate, respectively, for this experiment. The optimal flow rate of 6% t-butanol for the assay of zinc is 1.0 mL/min. This flow rate is an optimum since lower solvent flow rates will result in excessive burner head temperatures.

A set of 2 ppm copper standards was analyzed under fixed experimental conditions at various flow rates. The copper absorbances at the various flow rates were determined at 324.754 nm. The results of this experiment are listed in Table 1.3.9.3. The optimal flow rate of 6% t-butanol for the assay of copper is determined from the experimental data by selecting the maximal value from the calculation of the product of the absorbance times the inverse of the %RSD. Figures 1.3.9.4 and 1.3.9.5 are graphs of the copper absorbance versus flow rate and the %RSD versus flow rate, respectively, for this experiment. The optimal flow rate of 6% t-butanol for the assay of copper is 2.5 mL/min.

TABLE 1.3.9.1

Pressure in the Helium Reservoir versus Flow Rate

Helium, psi	Volume, mL	Time, min	Flow Rate, mL/min
8	3.0	3.04	1.0
14	3.0	1.78	1.7
20	5.0	1.97	2.5
30	7.0	1.79	3.9
40	7.0	1.32	5.3
50	8.0	1.18	6.8

TABLE 1.3.9.2

Zinc Absorbance and %RSD versus Flow Rate

Helium, psi	Flow Rate, mL/min	Absorbance, Zn	%RSD	Absorbance/%RSD
8	1.0	0.077	4.1	0.019
14	1.7	0.070	4.0	0.018
20	2.5	0.065	4.7	0.014
30	3.9	0.052	4.2	0.012
40	5.3	0.043	4.1	0.010
50	6.8	0.034	4.1	0.008

TABLE 1.3.9.3

Copper Absorbance and %RSD versus Flow Rate

Helium, psi	Flow Rate, mL/min	Absorbance, Cu	%RSD	Absorbance/%RSD
8	1.0	0.064	13.1	0.005
14	1.7	0.066	7.9	0.008
20	2.5	0.073	2.9	0.025
30	3.9	0.055	3.7	0.015
40	5.3	0.046	4.7	0.010
50	6.8	0.040	9.6	0.004

FIGURE 1.3.9.1

Pressure in the Helium Reservoir versus Flow Rate

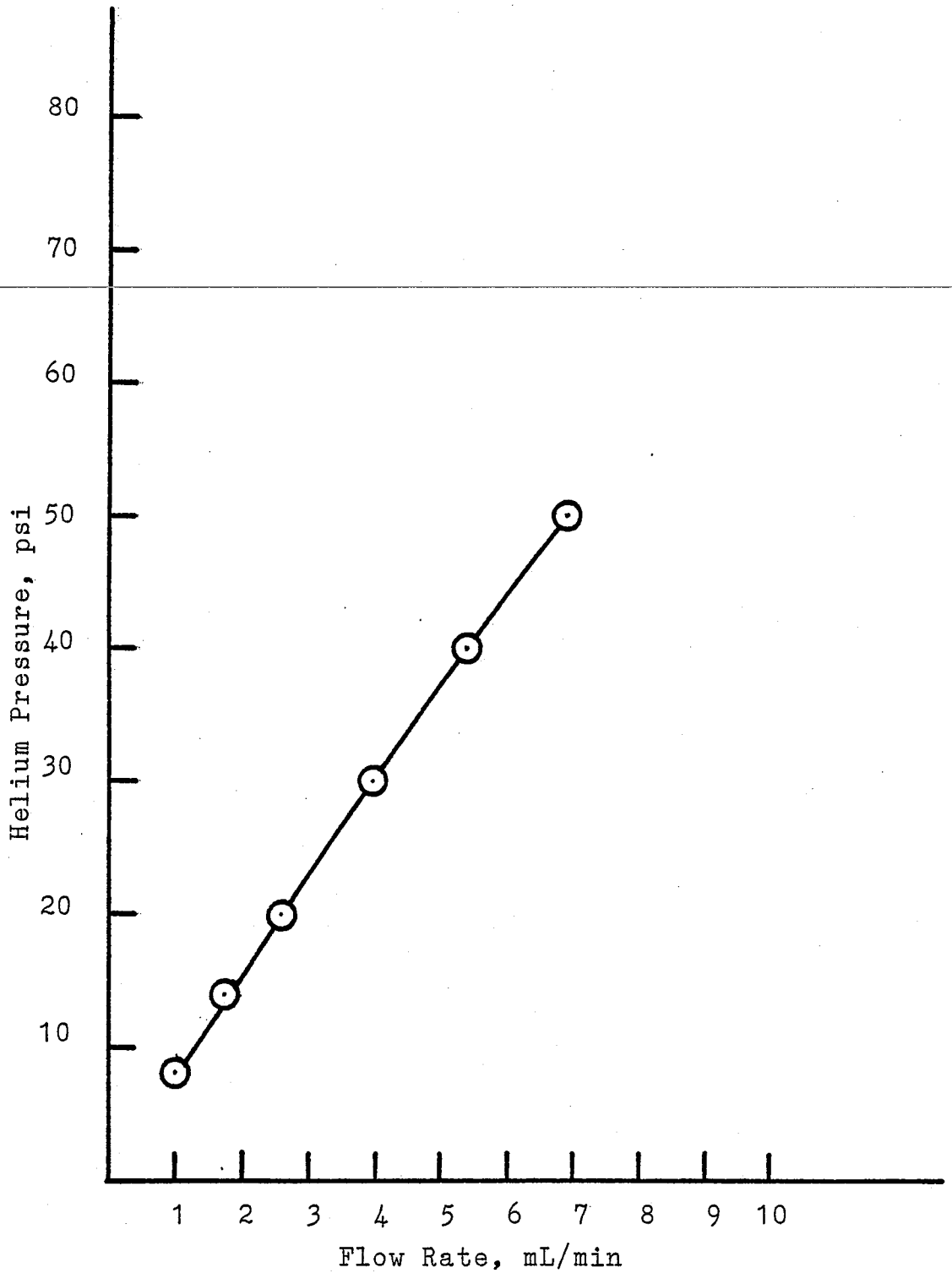

80
70
60
50
40
30
20
10
Helium Pressure, psi
1
2
3
4
5
6
7
8
9
10
Flow Rate, mL/min

FIGURE 1.3.9.2

Zinc Absorbance versus Flow Rate

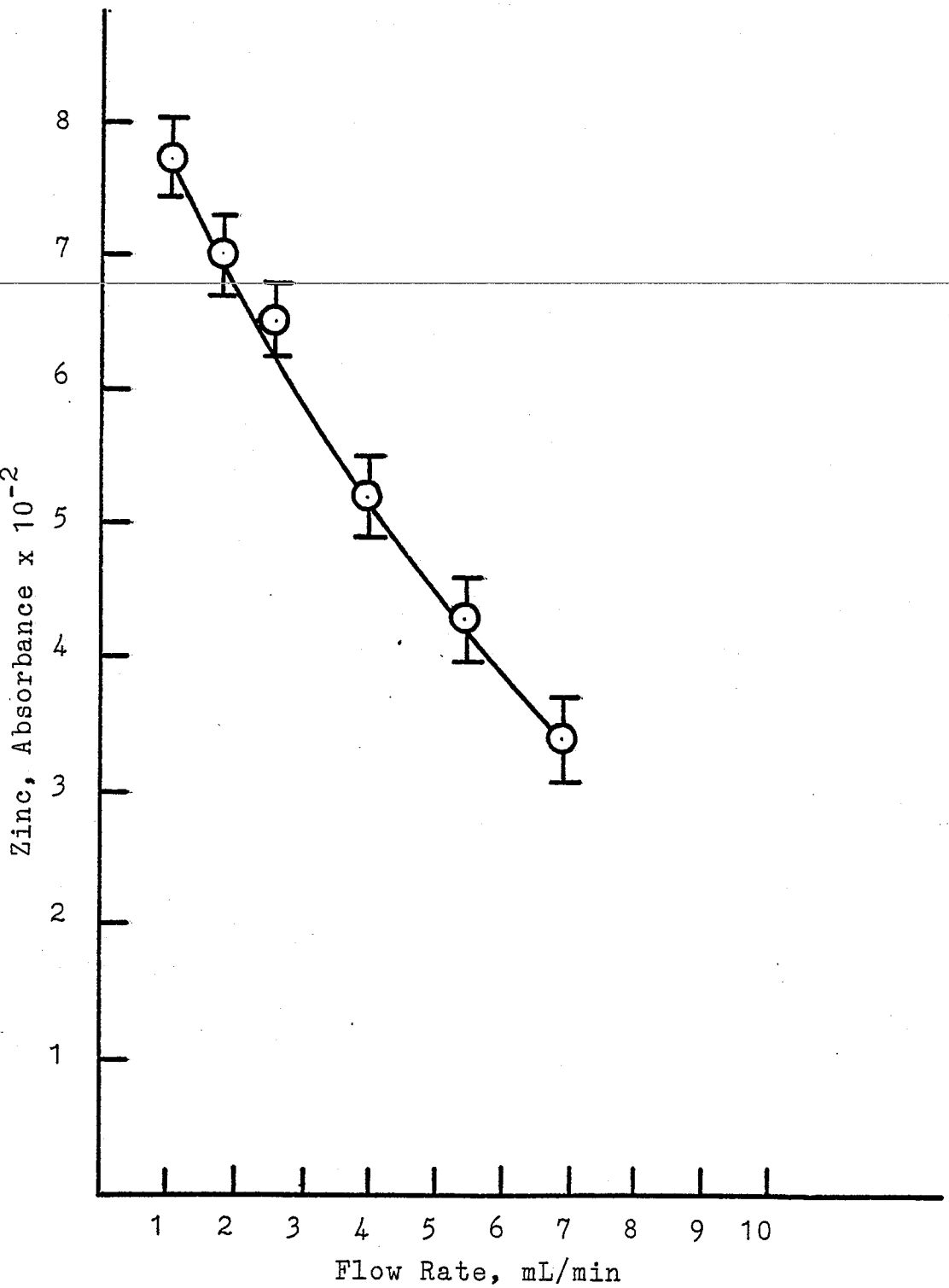

Zinc, Absorbance x 10^{-2}
8
7
6
5
4
3
2
1
1 2 3 4 5 6 7 8 9 10
Flow Rate, mL/min

FIGURE 1.3.9.3

Zinc %RSD versus Flow Rate

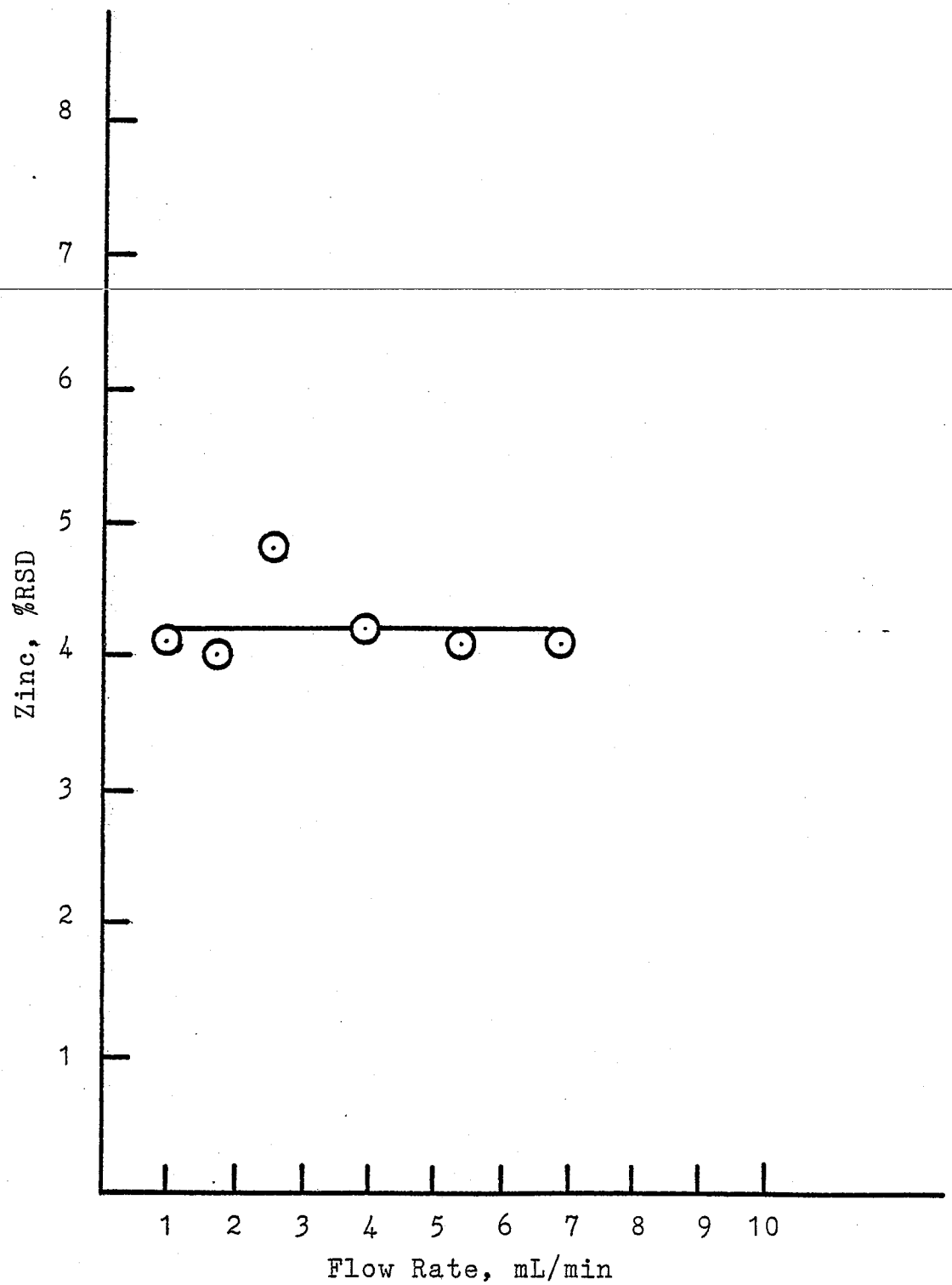

8
7
6
5
4
3
2
1
Zinc, %RSD
1
2
3
4
5
6
7
8
9
10
Flow Rate, mL/min

FIGURE 1.3.9.4

Copper Absorbance versus Flow Rate

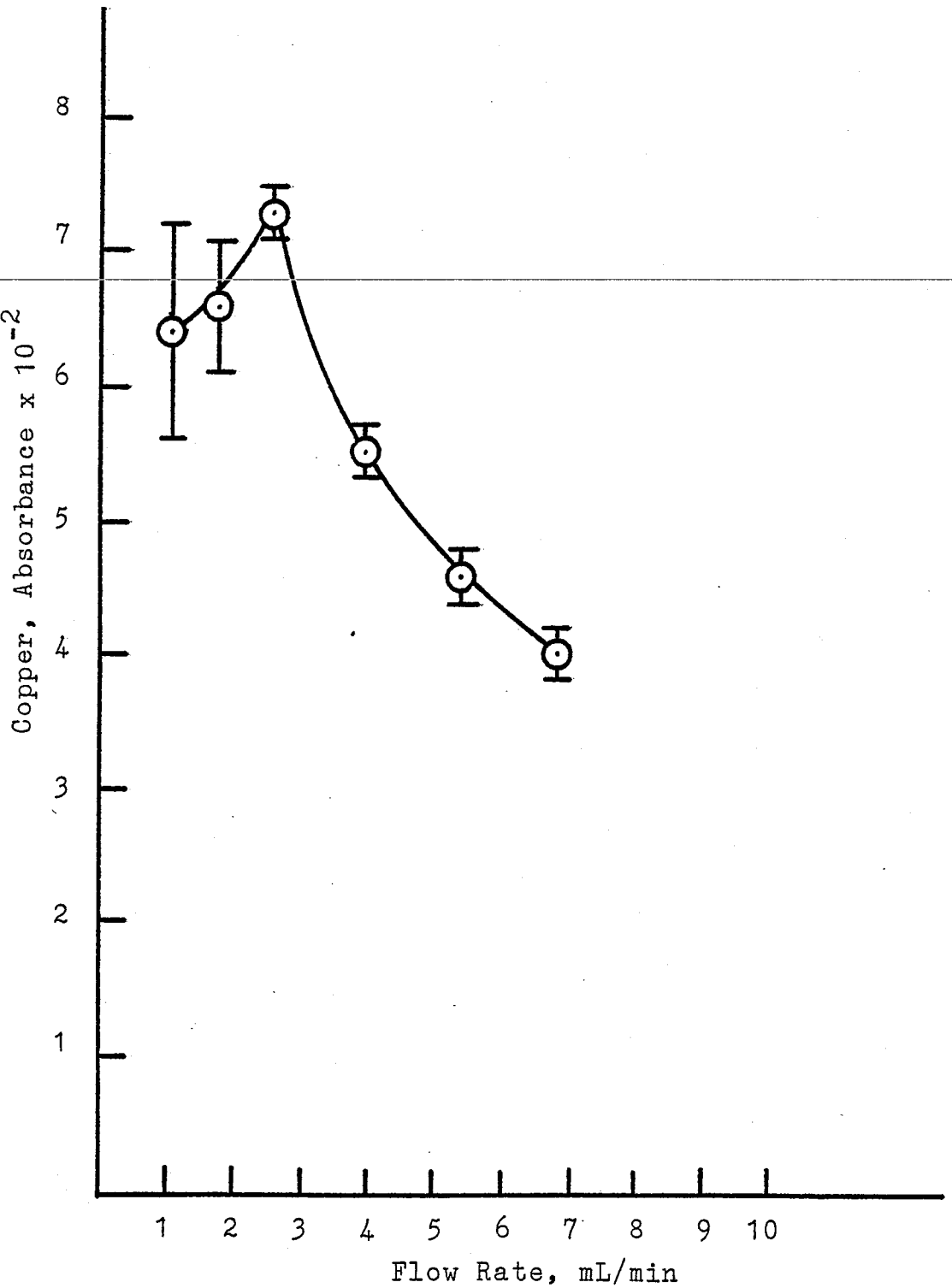
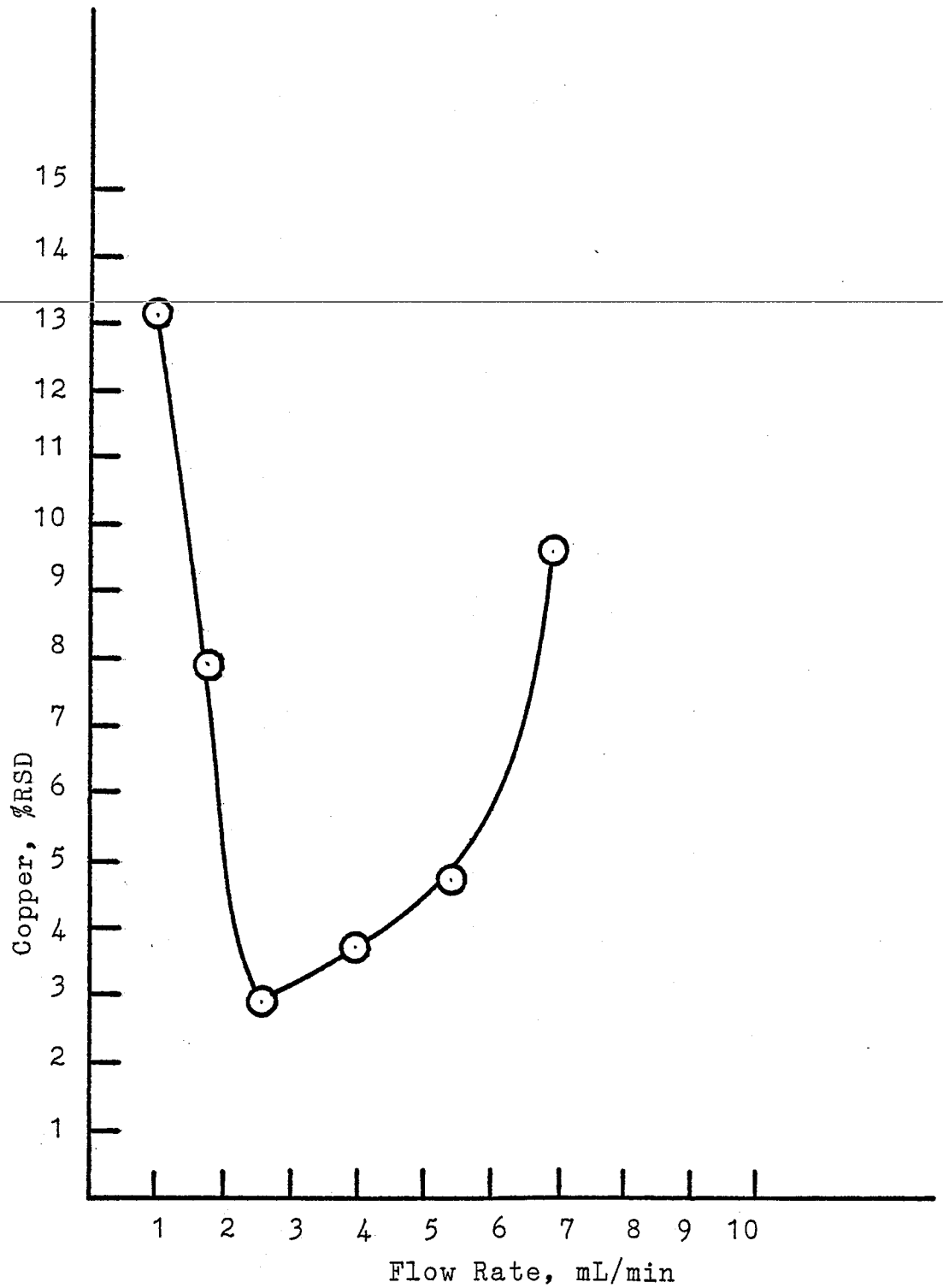

Copper, Absorbance x 10^{-2}
8
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
Flow Rate, mL/min

FIGURE 1.3.9.5

Copper %RSD versus Flow Rate


15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
Copper, %RSD
1
2
3
4
5
6
7
8
9
10
Flow Rate, mL/min

1.3.10 Matrix Matching of Standard Solutions

The copper and zinc concentrations in the mouse plasma samples were calculated from the absorbance data by a linear least squares method based upon calibration with aqueous copper and zinc standards. The validity of this use of aqueous metal standard solutions for the determination of concentrations of metals in very complicated sample matrices such as mouse plasma was established by a very extensive series of experiments involving copper and zinc aqueous and matrix matched standards and additions of such standards to pooled human plasma.

The standard curves, standards, and results are presented in the following tables and figures.

TABLE	FIGURE	
1.3.10.1	1.3.10.1	Matrix Matched Copper Standards
1.3.10.2	1.3.10.2	Standard Additions of Aqueous Copper Standards to Pooled Human Plasma
1.3.10.3	1.3.10.3	Standard Additions of Matrix Matched Copper Standards to Pooled Human Plasma

1.3.10.4	1.3.10.4	Matrix Matched Zinc Standards
1.3.10.5	1.3.10.5	Standard Additions of Aqueous Zinc Standards to Pooled Human Plasma
1.3.10.6	1.3.10.6	Standard Additions of Matrix Matched Zinc Standards to Pooled Human Plasma
1.3.10.7		Copper and Zinc Standard Solutions
1.3.10.8		Copper and Zinc Standard Solutions in Pooled Human Plasma
1.3.10.9		Slopes of Aqueous and Matrix Matched Standard Curves

The matrix matched standards consisted of the metal ion at the appropriate concentration, 0.15 M NaCl, and 6% Dextran(173,000 MW, Sigma) (20,22). The experiments consisted of obtaining the standard curves for the aqueous and matrix matched standards and the human plasma standard additions curves for the aqueous and matrix matched standards which had been added to the pooled human plasma. A comparison of the slopes of the various graphs is presented in Table 1.3.10.9.

The slope of an ideal matrix matched standard will be the same as the slope of a matrix matched standard additions curve. The matrix match is considered to be adequate when the slopes yield results that are, within the precision of the measurement, statistically indistinguishable.

This is the case for the aqueous zinc standards and the aqueous or the matrix matched additions to the pooled human plasma. The matrix matched zinc standards will yield results that are approximately 20% high. The validity of the use of aqueous copper standards is questionable. The use of matrix matched copper standards is preferred. The aqueous copper standards, however, produce results that are less than 5% high. This is well within the 10% RSD precision criteria at the 1 ppm level. Aqueous standards were used for the determination of copper and zinc in mouse plasma.

TABLE 1.3.10.1

Matrix Matched Copper Standards

Solution #	Cu, ppm	Absorbance	Standard Deviation	%RSD	n
9	5.00	0.0543	0.0019	3.5	9
10	2.00	0.0248	0.0012	4.9	10
11	1.00	0.0110	0.0012	11.1	10
12	0.50	0.0068	0.0006	9.3	9

Linear Least Squares

n	=	4
Correlation	=	0.9980
Slope	=	0.0106
x = 0, y	=	0.0016
y = 0, x	=	-0.1507

FIGURE 1.3.10.1

Matrix Matched Copper Standards

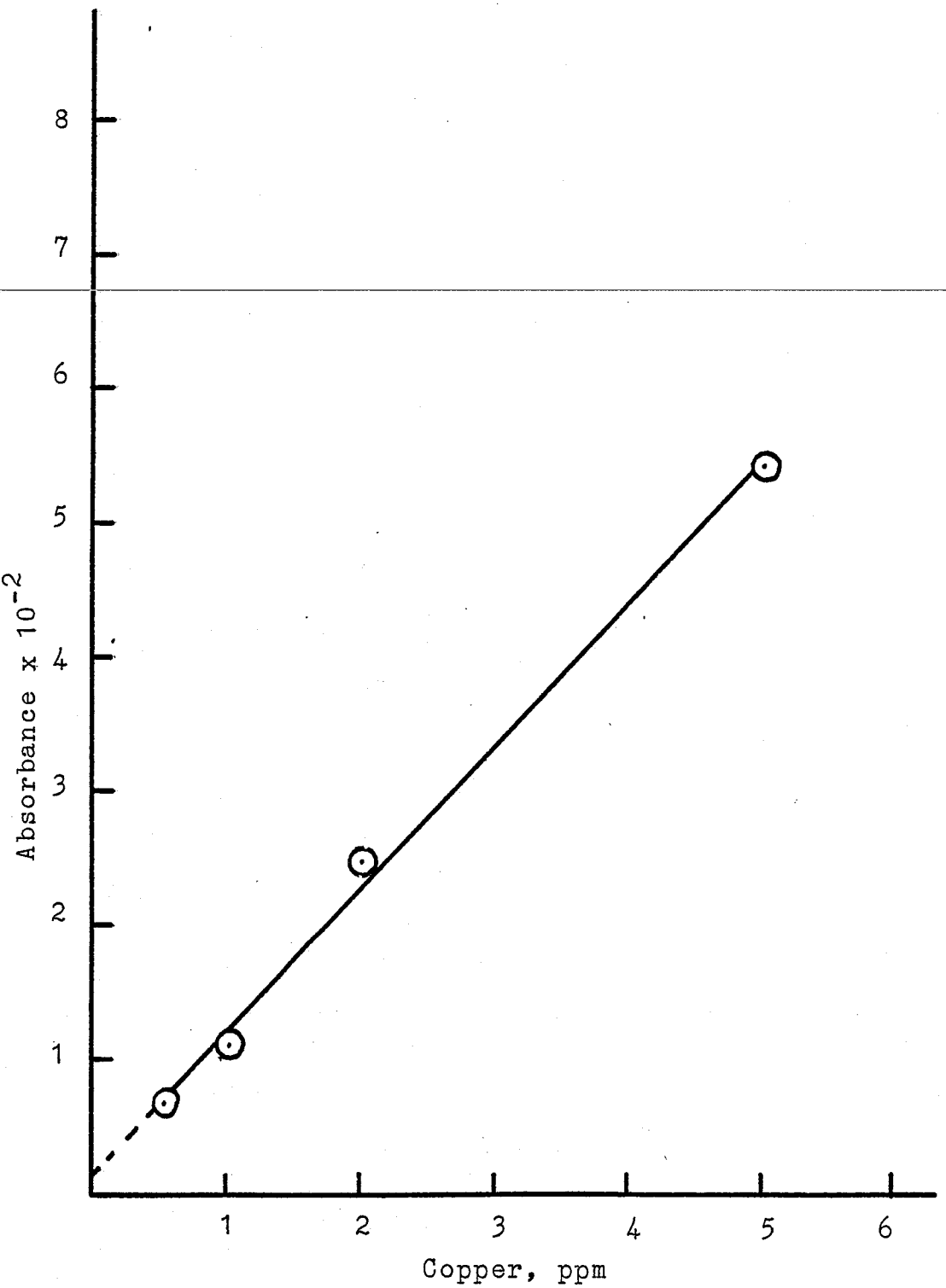

8
7
6
5
4
3
2
1
Absorbance x 10^{-2}
1
2
3
4
5
6
Copper, ppm

TABLE 1.3.10.2

Standard Additions of Aqueous Copper Standards to Pooled Human Plasma

Solution #	Cu, ppm	Absorbance	Standard Deviation	%RSD	n
(1,14)	0.00	0.0090	0.0011	12.6	10
(3,14)	0.50	0.0152	0.0008	5.1	10
(5,14)	0.99	0.0201	0.0010	5.0	10
(7,14)	1.96	0.0300	0.0017	5.6	10
(9,14)	4.76	0.0621	0.0013	2.0	10

Linear Least Squares

n	=	5
Correlation	=	0.9997
Slope	=	0.0111
x = 0, y	=	0.0091
y = 0, x	=	-0.82

FIGURE 1.3.10.2

Standard Additions of Aqueous Copper Standards to Pooled Human Plasma

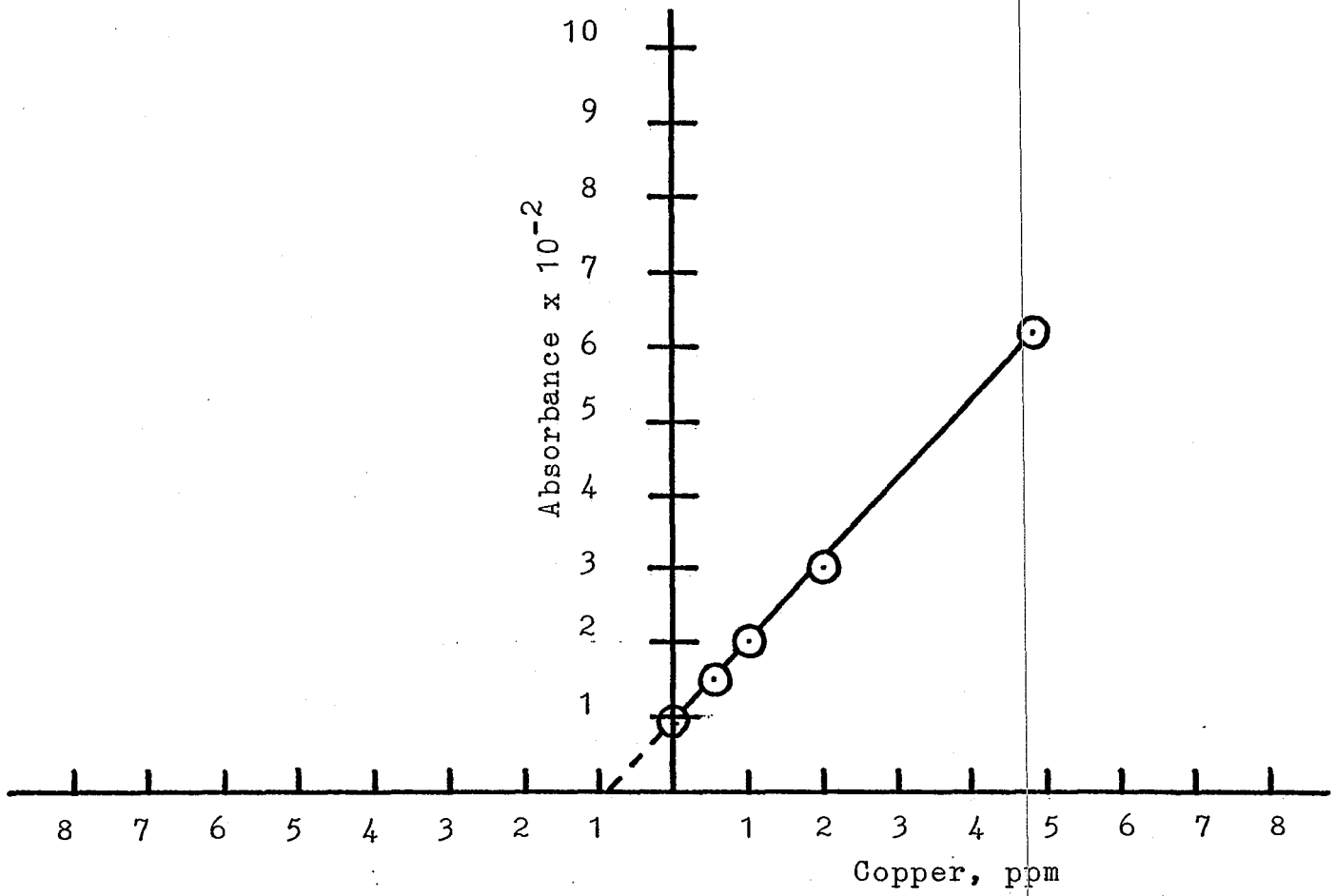

10
9
8
7
6
5
4
3
2
1
Absorbance x 10^{-2}
8 7 6 5 4 3 2 1
1 2 3 4 5 6 7 8
Copper, ppm

TABLE 1.3.10.3

Standard Additions of Matrix Matched Copper Standards to Pooled Human Plasma

Solution #	Cu, ppm	Absorbance	Standard Deviation	%RSD	n
(1,16)	0.00	0.0077	0.0012	15.6	10
(3,16)	0.50	0.0146	0.0011	7.5	10
(5,16)	0.99	0.0194	0.0008	4.0	10
(7,16)	1.96	0.0317	0.0009	3.0	10
(9,16)	4.76	0.0605	0.0012	2.0	10

Linear Least Squares

n	=	5
Correlation	=	0.9990
Slope	=	0.0110
x = 0, y	=	0.0087
y = 0, x	=	-0.79

FIGURE 1.3.10.3

Standard Additions of Matrix Matched Copper Standards to Pooled Human Plasma

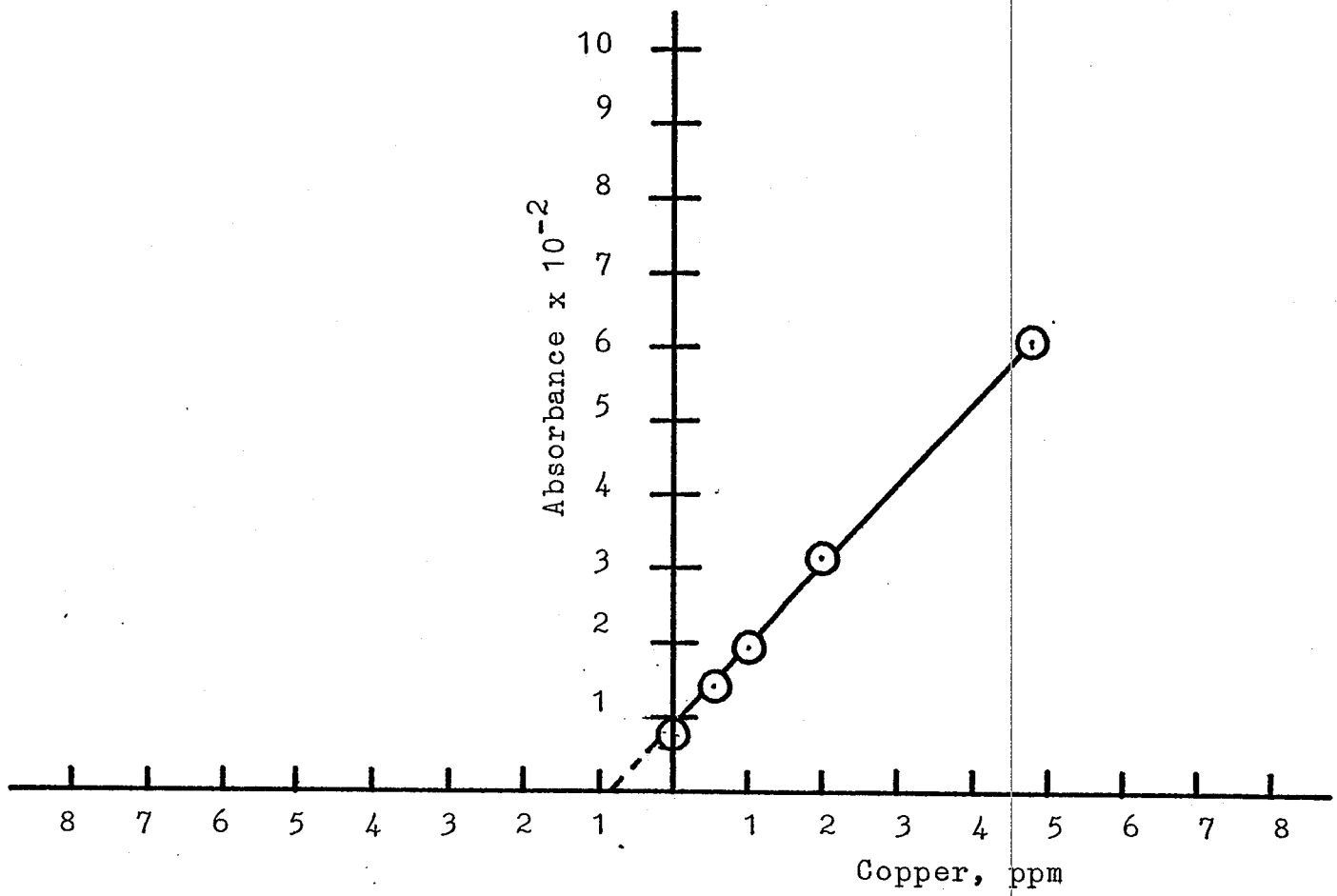

10
9
8
7
6
5
4
3
2
1
Absorbance x 10^{-2}
8 7 6 5 4 3 2 1
1 2 3 4 5 6 7 8
Copper, ppm

TABLE 1.3.10.4

Matrix Matched Zinc Standards

Solution #	Zn, ppm	Absorbance	Standard Deviation	%RSD	n
13	5.00	0.0933	0.0068	7.3	10
14	2.00	0.0531	0.0034	6.3	10
15	1.00	0.0316	0.0041	13.0	10
16	0.50	0.0195	0.0018	9.2	10

Linear Least Squares

n	=	3
Correlation	=	0.9996
Slope	=	0.0223
x = 0, y	=	0.0087
y = 0, x	=	-0.39

FIGURE 1.3.10.4

Matrix Matched Zinc Standards

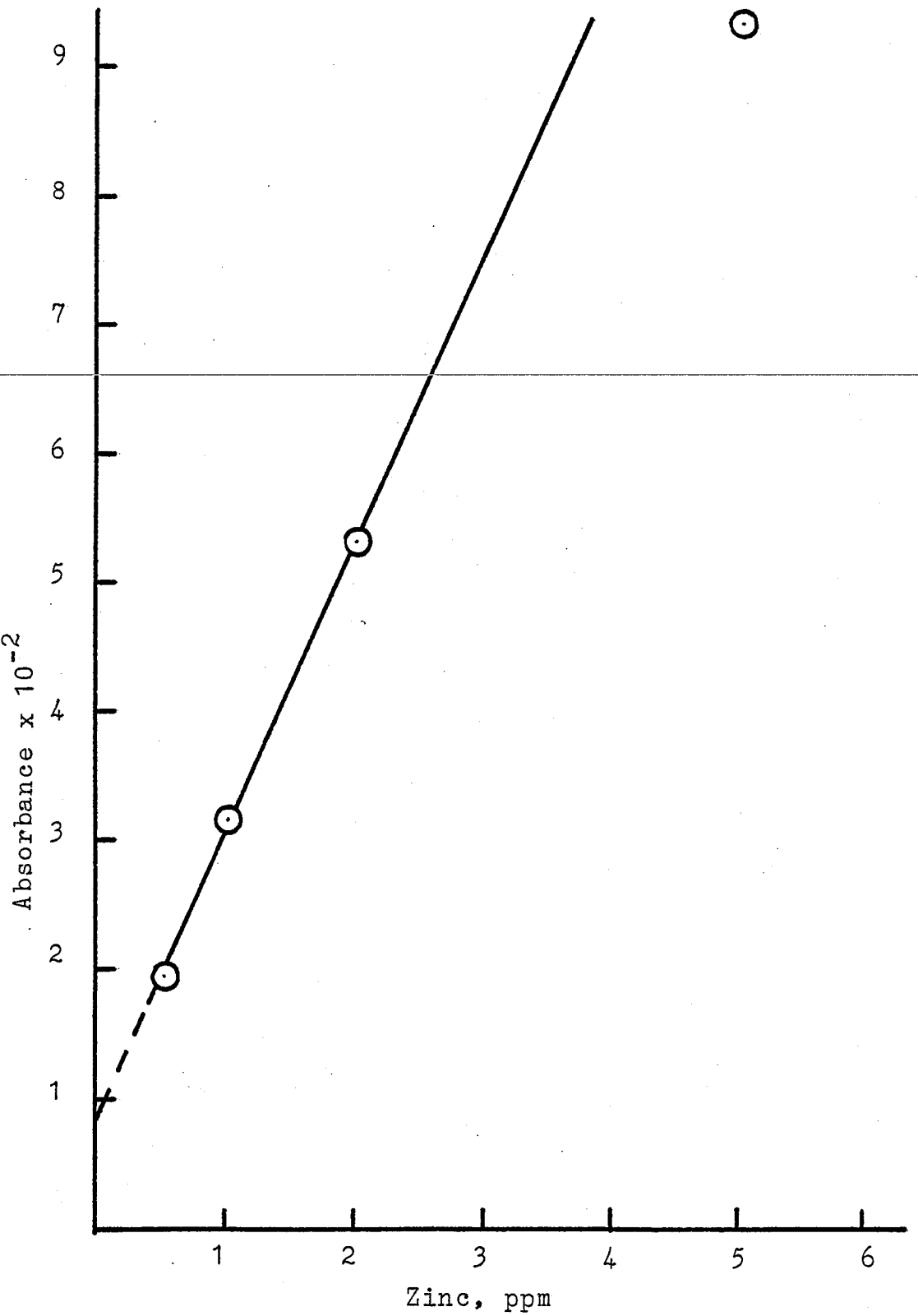

9
8
7
6
5
4
3
2
1
Absorbance x 10^{-2}
1
2
3
4
5
6
Zinc, ppm

TABLE 1.3.10.5

Standard Additions of Aqueous Zinc Standards to Pooled Human Plasma

Solution #	Zn, ppm	Absorbance	Standard Deviation	%RSD	n
(1,11)	0.00	0.0321	0.0034	10.7	10
(3,11)	0.50	0.0449	0.0021	4.6	10
(5,11)	0.99	0.0535	0.0051	9.6	10
(7,11)	1.96	0.0709	0.0047	6.6	10
(9,11)	4.76	0.1034	0.0037	3.5	10

Linear Least Squares

n	=	4
Correlation	=	0.9963
Slope	=	0.0194
x = 0, y	=	0.0336
y = 0, x	=	-1.70

FIGURE 1.3.10.5

Standard Additions of Aqueous Zinc
Standards to Pooled Human Plasma

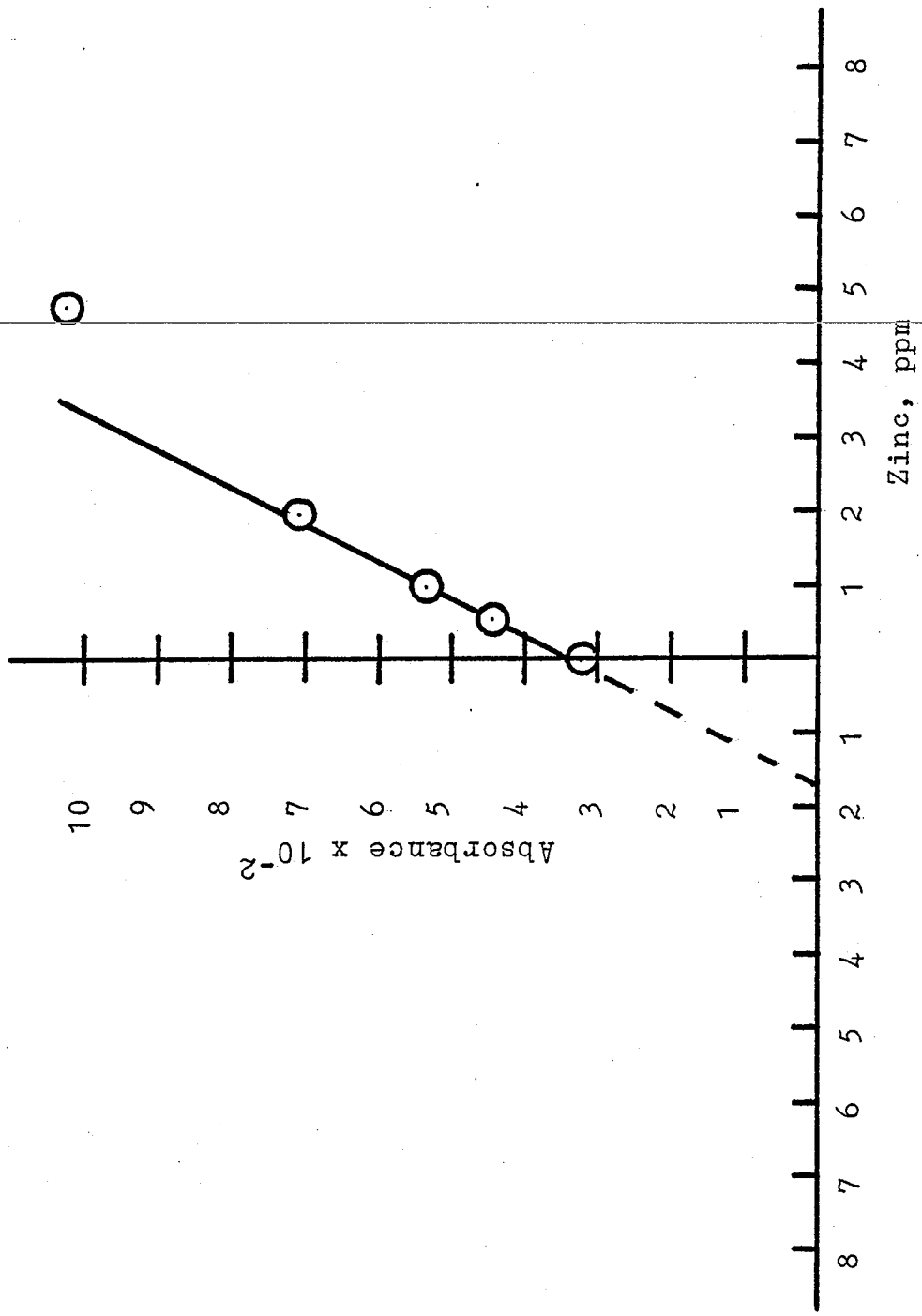

Absorbance x 10-2
10 9 8 7 6 5 4 3 2 1
1 2 3 4 5 6 7 8
Zinc, ppm

TABLE 1.3.10.6

Standard Additions of Matrix Matched Zinc Standards to Pooled Human Plasma

Solution #	Zn, ppm	Absorbance	Standard Deviation	%RSD	n
(1,13)	0.00	0.0317	0.0026	8.3	9
(3,13)	0.50	0.0452	0.0043	9.5	10
(5,13)	0.99	0.0558	0.0047	8.4	10
(7,13)	1.96	0.0714	0.0049	6.9	10
(9,13)	4.76	0.1011	0.0039	3.9	10

Linear Least Squares

n	=	4
Correlation	=	0.9918
Slope	=	0.0199
x = 0, y	=	0.0338
y = 0, x	=	-1.70

FIGURE 1.3.10.6

Standard Additions of Matrix Matched Zinc Standards to Pooled Human Plasma

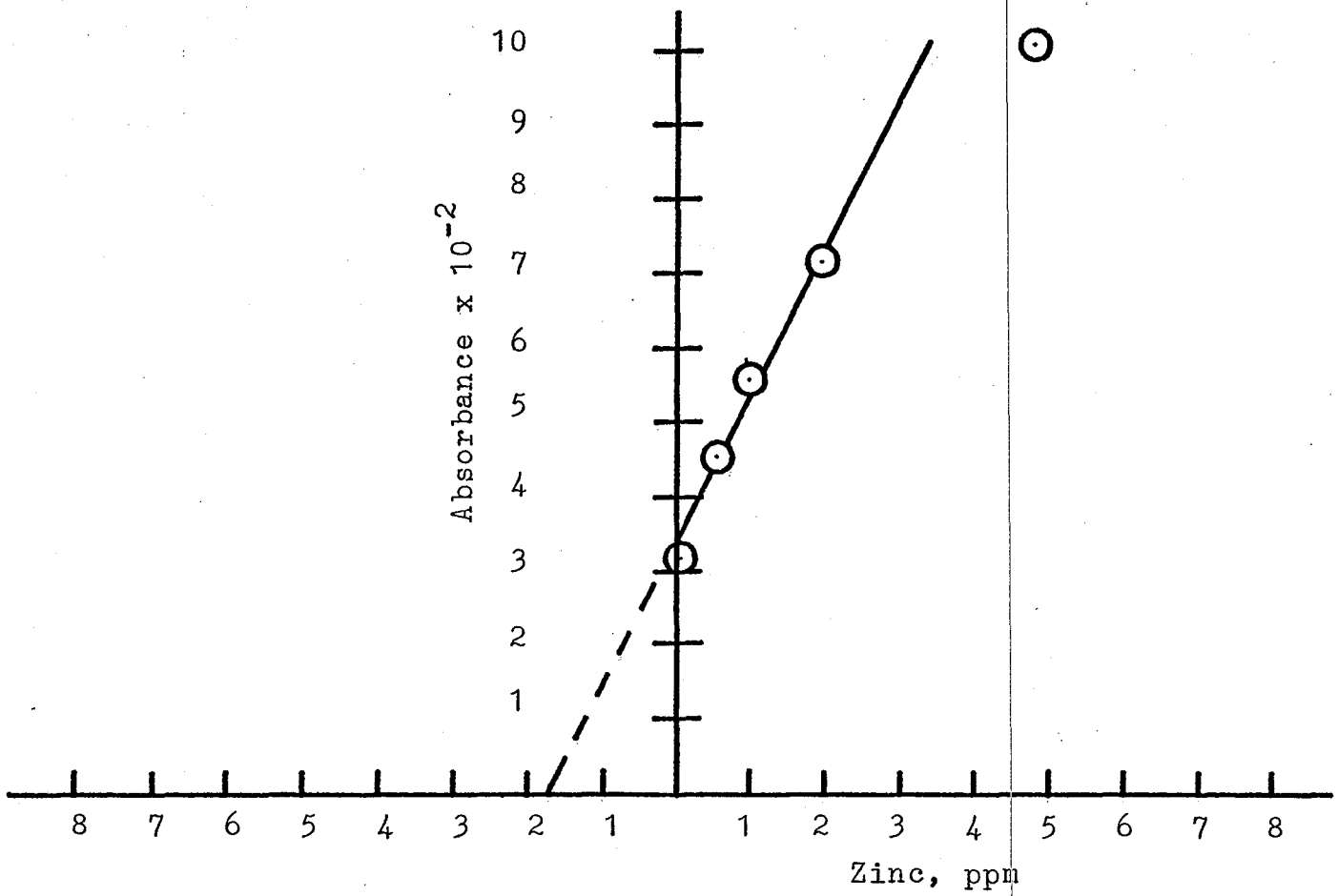

10
9
8
7
6
5
4
3
2
1
Absorbance x 10^{-2}
8 7 6 5 4 3 2 1 1 2 3 4 5 6 7 8
Zinc, ppm

TABLE 1.3.10.7

Copper and Zinc Standard Solutions

Solution #	Cu, ppm	Zn, ppm	NaCl, M	Dextran, %(v/v)
1	5.00			
2	2.00			
3	1.00			
4	0.50			
5		5.00		
6		2.00		
7		1.00		
8		0.50		
9	5.00		0.15	6
10	2.00		0.15	6
11	1.00		0.15	6
12	0.50		0.15	6
13		5.00	0.15	6
14		2.00	0.15	6
15		1.00	0.15	6
16		0.50	0.15	6

TABLE 1.3.10.8

Copper and Zinc Standard Solutions
in Pooled Human Plasma

Solution #	Cu, ppm	Zn, ppm	Added NaCl $\times 10^{-3}$M	Added Dextran %(v/v)
(1,11)		0.00		
(3,11)		0.50		
(5,11)		0.99		
(7,11)		1.96		
(9,11)		4.76		
(1,13)		0.00	0.00	0.00
(3,13)		0.50	0.75	0.03
(5,13)		0.99	1.49	0.06
(7,13)		1.96	2.94	0.12
(9,13)		4.76	7.14	0.29
(1,14)	0.00			
(3,14)	0.50			
(5,14)	0.99			
(7,14)	1.96			
(9,14)	4.76			

TABLE 1.3.10.8 (CONTINUED)

Solution #	Cu, ppm	Zn, ppm	Added NaCl _ x 10^{-3}M	Added Dextran %(v/v)
(1,16)	0.00		0.00	0.00
(3,16)	0.50		0.75	0.03
(5,16)	0.99		1.49	0.06
(7,16)	1.96		2.94	0.12
(9,16)	4.76		7.14	0.29

TABLE 1.3.10.9

Slopes of Aqueous and Matrix Matched Standard Curves

Table	Slope	Metal	Standard
~~1.5.1~~	~~0.0126~~	~~Cu~~	~~Aqueous~~
1.3.10.1	0.0106	Cu	Matrix Matched
1.3.10.2	0.0111	Cu	Aqueous Additions, Plasma
1.3.10.3	0.0110	Cu	Matrix Additions, Plasma
1.5.2	0.0198	Zn	Aqueous
1.3.10.4	0.0223	Zn	Matrix Matched
1.3.10.5	0.0194	Zn	Aqueous Additions, Plasma
1.3.10.6	0.0199	Zn	Matrix Additions, Plasma

1.4 Instrumental Parameters for Atomic Absorption

A summary of the instrumental parameters for atomic absorption as developed in section 1.3 is given in Table 1.4.1. These flow injection analysis system parameters were used for the assay of copper and zinc in mouse plasma with the Perkin-Elmer Model 303 atomic absorption spectrometer.

TABLE 1.4.1

Instrumental Parameters for Atomic Absorption

Parameter	Copper	Zinc
Wavelength	324.754 nm	213.856 nm
Recorder Chart Speed	1 cm/min	1 cm/min
Recorder mv	20 mv	50 mv
Recorder Readout Noise	2x	2x
Recorder Readout Scale	30x	30x
Slit	4	5
Helium Pressure on the Solvent Reservoir	20 psi	8 psi
Solvent Flow Rate	2.5 mL/min	1.0 mL/min
Flow Injection Analysis Solvent	6%(v/v) t-butanol in Water	6%(v/v) t-butanol in Water
Burner Head Type	Triple Slot	Triple Slot
Burner Lead Head Screw Turns ccw	1.75	1.75
Burner Height	-1.5 cm	-4.0 cm
Fuel Flow	9.5(C_2H_2)	5.0(H_2)
Oxidant Flow	9.5(Air)	9.0(Argon)
Injection Size	10 µL	10 µL

1.5 Standard Calibration Curves for Copper and Zinc

TABLE 1.5.1

Absorbance of Aqueous Copper Standards

Cu, ppm	Absorbance	Standard Deviation	%RSD	n
5.00	0.0627	0.0013	2.1	11
2.00	0.0252	0.0007	2.8	10
1.00	0.0123	0.0008	6.6	10
0.50	0.0062	0.0009	15.2	10

Linear Least Squares

n	=	4
Correlation	=	1.0000
Slope	=	0.0126
x = 0, y	=	-0.0001
y = 0, x	=	0.0090

FIGURE 1.5.1

Absorbance versus Concentration of Copper Standards

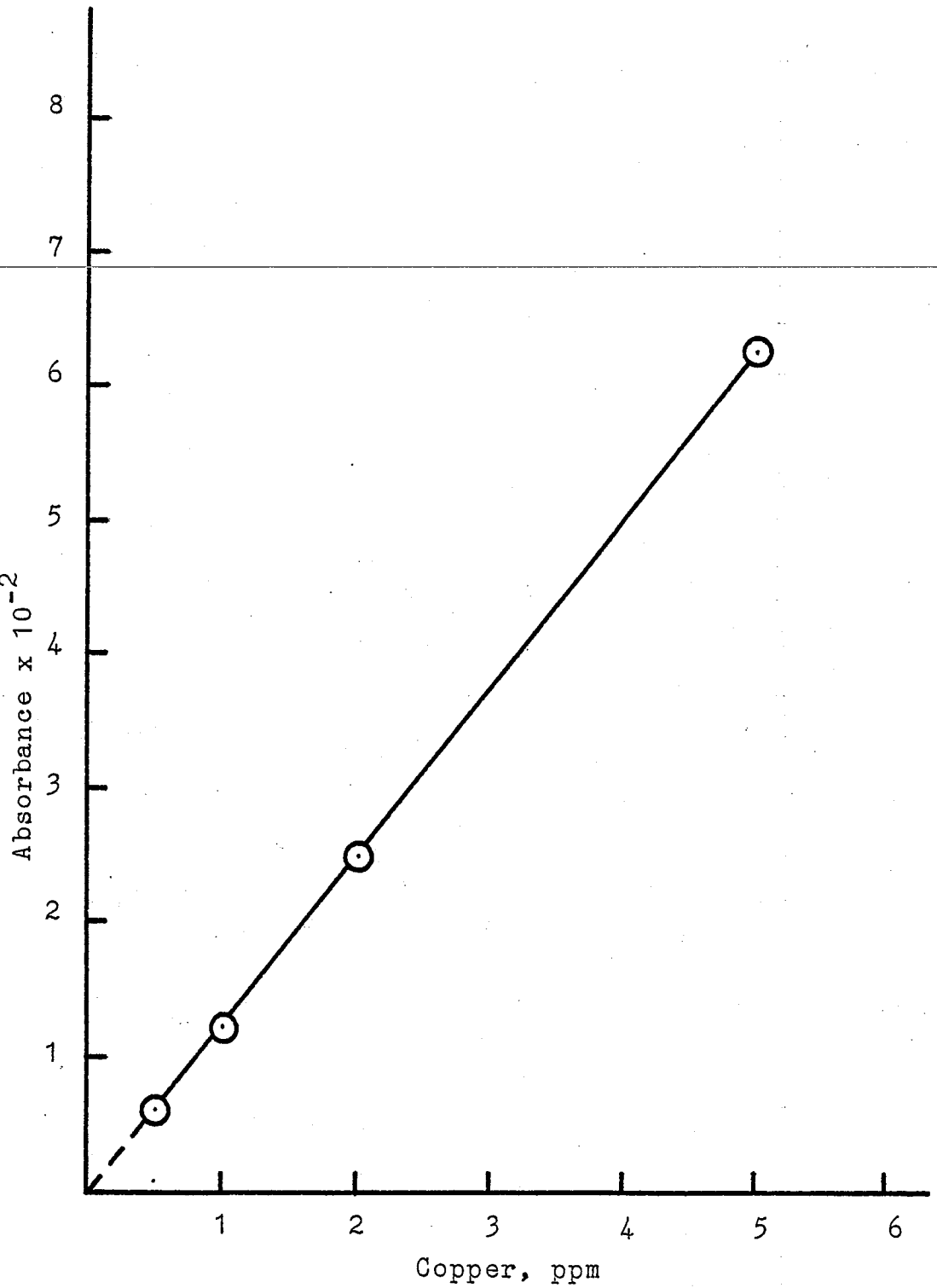

8
7
6
5
4
3
2
1
Absorbance x 10^{-2}
1
2
3
4
5
6
Copper, ppm

TABLE 1.5.2

Absorbance of Aqueous Zinc Standards

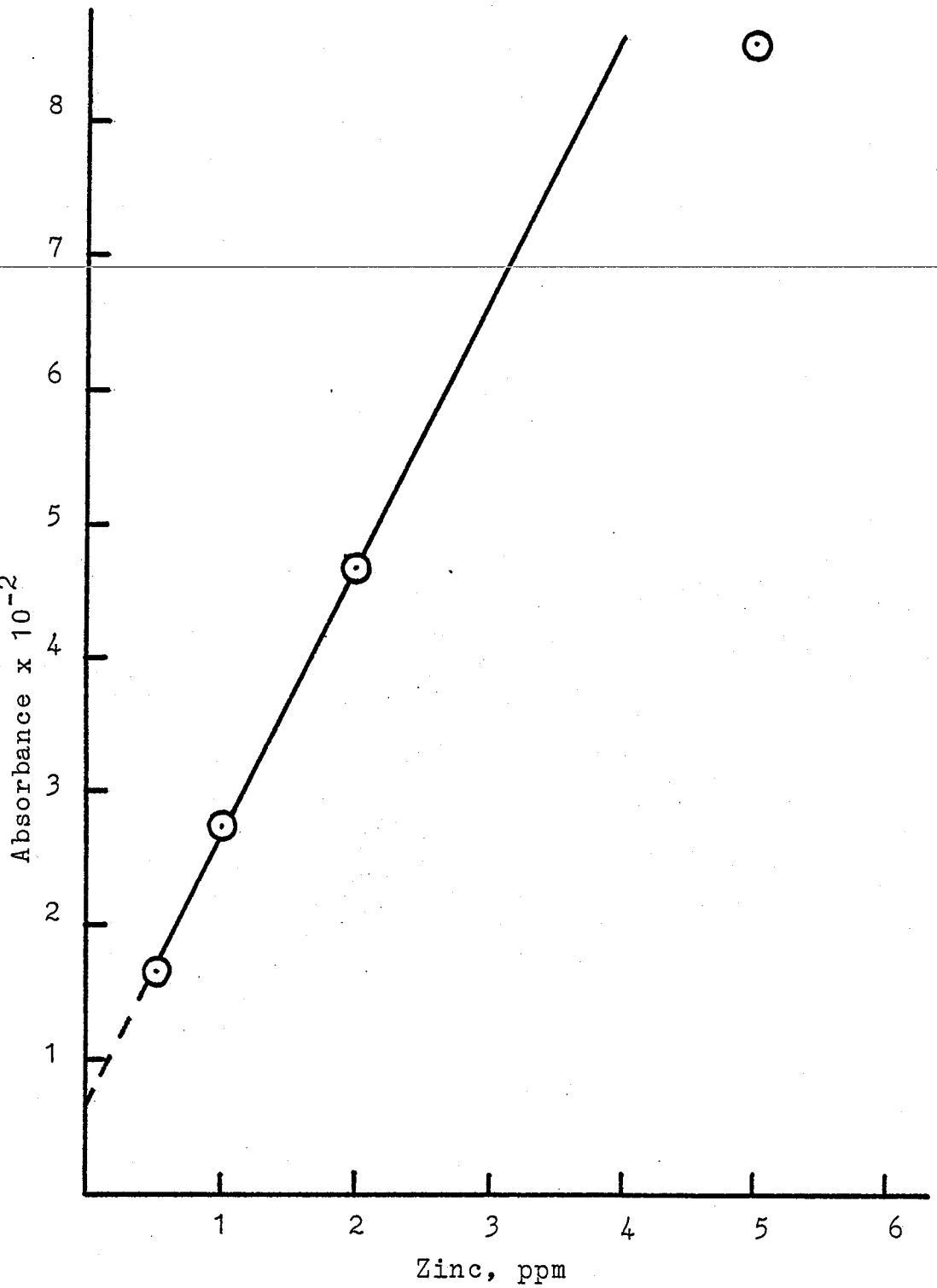
Zn, ppm	Absorbance	Standard Deviation	%RSD	n
5.00	0.0859	0.0028	3.3	10
2.00	0.0464	0.0029	6.3	10
1.00	0.0273	0.0018	6.6	10
0.50	0.0165	0.0011	6.6	10

Linear Least Squares

n	=	3
Correlation	=	0.9994
Slope	=	0.0198
x = 0, y	=	0.0069
y = 0, x	=	-0.3499

FIGURE 1.5.2

Absorbance versus Concentration of Zinc Standards


8
7
6
5
4
3
2
1
Absorbance x 10^{-2}
1
2
3
4
5
6
Zinc, ppm

1.6 Data Analysis Programs and Procedures

1.6.1 Program to Calculate Absorbance Values from Peak Height

The Perkin-Elmer Model 303 atomic absorption spectrometer produces a readout value in absorption, which may be simultaneously obtained from the recorder readout accessory for display on a stripchart recorder. The analysis system produces a " gas chromatograph " type of peak signal on the stripchart recorder. The peak height is proportional to the absorption value. Peak heights in mm are converted into their equivalent absorbance value by the use of the logarithmic relationship between absorption and absorbance, Absorption = 100 - Transmission and Absorbance = $-\log_{10}$(Transmission).

For this conversion, the boundary conditions in mm which correspond to the 0 and 100 percent absorption signals must be determined. The 0 percent absorption signal is a peak height of 0 mm. If 100 mm had been chosen as the boundary condition to represent 100 percent absorption there would have been a direct correlation between these absorbance values and those calculated from the instrument absorption values.

The problem which arises under the 100 mm boundary condition is that we are restricted to only using a 100 mm section of a 250 mm wide chart paper. The decision was to use the full width of the chart paper by adopting a 1000 mm boundary condition. The following program was used to convert peak heights in mm to absorbance with the boundary condition that 1000 mm is equal to 100 percent absorption. This program is written for a Texas Instruments Model 55 (TI-55) calculator. It is based upon the formula of Absorbance = $-\log_{10}((1000 - mm) / 1000)$.

2nd CA 2nd Fix 6 2nd Lrn ± + 1000 = ÷ 1000 = 2nd log = ± = 2nd R/S 2nd Rst 2nd Lrn 2nd Rst

An earlier equivalent formula and its corresponding program is Absorbance = $-\log_{10}((100 - (mm \times 10^{-1})) / 100)$.

2nd CA 2nd Fix 6 2nd Lrn x (1 ± 2nd 10^x) = ± + 100 = ÷ 100 = 2nd log = ± = 2nd R/S 2nd Rst 2nd Lrn 2nd Rst

Program verification:

To determine the absorbance of a peak height in mm, do the following:

1.	Enter the height in mm	ex: 14.5	ex: 18.5
2.	Push 2nd		
3.	Push R/S		
4.	The register will read	0.006343	0.008110

1.6.2 Program to Calculate Absorbance Values from Absorption Values

The following program was written for a TI - 55 calculator. The program permits the calculation of absorbance values from absorption values. An absorption value of 1.0000 is 100 percent absorption. The program is based upon the formula of absorbance = $\log_{10}$ (1 - absorption).

2nd CA 2nd Fix 4 2nd Lrn = ± + 1 = 2nd log = ± =
2nd R/S 2nd Rst 2nd Lrn 2nd Rst

Program verification:

To determine the absorbance from an absorption value, do the following:

1.	Enter the absorption value	ex: 0.4268	ex: 0.5000
2.	Push 2nd		
3.	Push R/S		
4.	The register will now read	0.2417	0.3010

1.6.3 Program to Calculate Peak Heights from Absorbance Values

The following program was written for a TI - 55 calculator. The program permits the back calculation of peak heights in mm from previously calculated absorbance(A) values where those absorbance values were calculated by the formula of absorbance = $- \log_{10}$ ((1000 - mm)/ 1000). The boundary condition is that 1000 mm of peak height is equal to 100 percent absorption. The program is based upon the formula of mm = (((1000 x 10^{A}) - 1000)/ 10^{A}).

2nd CA 2nd Fix 0 2nd Lrn STO 1 (RCL 1 2nd 10^{x} x 1000 = - 1000 =) ÷ (RCL 1 2nd 10^{x}) = 2nd R/S 2nd Rst 2nd Lrn 2nd Rst

Program verification:

To determine the peak height in mm from a previously calculated absorbance value, do the following:

1. Enter the absorbance value ex: 0.068542 ex: 0.004804
2. Push 2nd
3. Push R/S
4. The register will now read 146 11

1.6.4 Procedures and Criteria for Data Analysis

The primary atomic absorption data from the experiments consisted of narrow peaks on stripchart recording paper. The height of each individual peak was determined with a metric ruler to the nearest mm. This height represents and is proportional to the size of the instrument absorption signal. An absorbance value was calculated from this peak height. The boundary condition in the calculation was that 1000 mm of peak height is equal to 100 percent absorption.

A linear least squares standard curve was prepared from the calculated absorbance values for the aqueous standards. Each standard was injected approximately ten times. Standards were injected at the beginning of every analysis session. The calculated absorbance values for the mouse plasma samples were then entered into the calculator. The concentration of the metal in ppm was determined from standard curves previously developed by a linear least squares method. A typical analysis run consisted of a series of four standards of the metal of interest and approximately fifty plasma samples. The total number of injections in a typical run was approximately ninety.

Data of questionable quality were individually rejected from the set of data when they fell outside of the 4Q criterium which requires the removal of data points that are away from the mean by more than four times the standard deviation. The mean and the standard deviation about the mean are calculated from the set of data remaining after the datum point in question has been removed. Data not obtained under the specified instrumental parameters, particularily the requirement for 10 µL of sample, were also removed.

CHAPTER TWO

2. Audiogenic Seizure Susceptible Mouse Model of Epilepsy

The analytical methods that were developed in Chapter One have been applied to an epilepsy project. That project, which involves the use of audiogenic seizure susceptible mice, is introduced in this chapter.

Epilepsy is a "recurrent self-sustained paroxysmal disorder of brain function characterized by excessive discharge of cerebral neurons" (23). The frequency of epilepsy in the human population is approximately 0.5 percent (24). Since the causes of epilepsy are as varied as the clinical expressions we prefer to view the manifestations of epilepsy as a symptom of one or more disease states.

Historically, epilepsy has long been recognized as a disease state in such documents as the Code of Hammurabi who set rules regarding the marriage of epileptics and the admissibility of their court testimony (24). Hippocrates considered epilepsy a disease of the brain (24).

Charles le Pois was the first to state that all epilepsies originate in the brain (24). Jackson introduced the idea of a discharging focus when he explained seizures as the result of paroxysmal discharges beginning at and spreading from a focus location in the brain (24). Berger introduced electroencephalography(EEG) (24). This technique is the recording of the millivolt potential of an epileptic discharge on a millisecond time scale. Such techniques make it possible to locate and characterize the epileptic discharge and also to develop and verify experimental models of human epilepsy by comparison of the resulting EEG seizure patterns.

Numerous models of human epilepsy have been developed and verified by EEG techniques for purposes of testing anticonvulsant drugs and studying the underlying chemistry of the epileptic disease state. A "model" of human epilepsy consists of a procedure applied to a certain animal or tissue. Models of human epilepsy which have been developed for the purpose of testing anticonvulsant drugs consist of procedures which involve the topical application of convulsant metals to neural tissue, freezing of a localized section of brain, administration of convulsant drugs to mammals, electrical stimulation of neural tissue, and disturbances of metabolic equilibria.

Models of human epilepsy which have been developed for the purpose of studying the underlying chemistry of the disease state consist of applications of the previously listed procedures, the application of those procedures to humans and other genetically seizure susceptible mammals, and procedures which involve the chemistry of genetically seizure susceptible mammals. These mammals are certain strains of goats, Senegal baboons, mice, rats, and rabbits (23, 25-30). All of these animals are susceptible to drug induced seizures. They are also susceptible to seizures which have been initiated by rather rare methods. Certain strains of Senegal baboons are seizure susceptible to intermittent light stimulation. Selected populations and strains of rabbits, rats, and mice are susceptible to audiogenic seizures. The advantages of low cost, availability of many different inbred strains, rapid reproductive rates, and the superb genetic definition obtained as a result of hundreds of generations of inbreeding has resulted in the establishment and widespread acceptance of the audiogenic seizure susceptible mouse model of epilepsy (28).

This model of epilepsy has two important experimental parameters. The first is that the percentage seizure susceptibility of mice is age dependent.

Vicari extablished the bimodal age dependence of the DBA/2 strain (31). Table 2.1 presents her results for the percent of DBA/2 mice by age that will convulse on the first trial within 90 seconds after sound initiation from an 80 to 90 db electric bell in a metal tub. She obtained a maximum of 90 percent seizure susceptibility for DBA/2 mice in the 30-34 day age group and a second maximum of 25 percent in the 60-69 day age group. Not all strains of seizure susceptible mice exhibit a bimodal susceptibility curve but they do have a curve that generally shows seizure susceptibility beginning around day 14, peaking in the 30's, and then declining (32,33).

The second important experimental parameter is that audiogenic seizure susceptibility varies widely among various inbred strains. Fuller and Sjursen have ranked the seizure susceptibility of eleven strains of inbred mice (34), in the order DBA/2J > LP/J > 129/J > RF/J > AKR/J > LG/J > SJL/J > CBA/J > SM/J > BALB/cJ > C57BL/6J. We obtained a similar but slightly different ranking of six of their strains by converting their graphical data into percentage seizure susceptibility by week, obtaining the average of the seizure susceptibility over the weeks 3,4,5, and 6 and ranking the six selected strains in the order of percentage seizure susceptibility.

TABLE 2.1

BDA/2 Mouse Age versus Percent Seizure Susceptibility

Mouse Age, Days	Percent Seizure Susceptibility
10-19	0
20-24	60
25-29	88
30-34	90
35-39	80
40-44	61
45-49	66
50-59	7
60-69	25
70-79	17
80-89	0
90-99	0
100-600	0

The result is the slightly modified percentage seizure susceptibility order of LP/J(97%) > DBA/2J(94%) > SJL/J(70%) > LG/J(65%) > BALB/cJ(24%) > C57BL/6J(14%). The combination of age variation and interstrain variation in seizure susceptibility allows the design of experiments showing the presence or absence of correlation between a measurable parameter and seizure susceptibility.

The transmission of seizure susceptibility from a seizure susceptible mouse to a non-seizure susceptible mouse by connecting their circulatory systems by surgical parabiotic union was demonstrated by Hamburgh(35). This experiment established the influence of some unknown aspect of the circulatory system such as the presence, absence, or a concentration change in a chemical upon seizure susceptibility. The experiment suggests that there exist significant chemical differences between the circulating fluids of susceptible and resistant mice.

The large number of metalloenzymes that contain copper and zinc, their influence in numerous metabolic pathways, the 25 to 40 percent elevation of copper and the 20 percent reduction of zinc in the serum of human epileptics receiving anticonvulsant medication, and the lack of data for copper and zinc plasma concentrations as a function of age for epileptic mice, led to considering this research project (36,37,38).

The project was to determine the plasma copper and zinc concentrations by age for the previously described series of six selected strains of audiogenic seizure susceptible mice, to compare the results with their age and strain dependent audiogenic seizure susceptibility, and to determine if there is any correlation between the seizure susceptibility of the mice and their plasma copper and/or zinc concentrations.

The experiments required repeated measurements of copper and zinc concentrations in 10 µL samples of mouse plasma with adequate precision at the one ppm level. This resulted in the extensive development of atomic absorption flow injection techniques to obtain the required accuracy and precision. For the necessary observation of a 20 percent change in zinc concentration, an analytical precision of 10 percent at the one ppm level was considered appropriate.

CHAPTER THREE

3. Experimental

3.1 Animal Husbandry

3.1.1 Mouse Cages and Racks

Six strains of non-pedigree littermate breeder mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. The strains were C57BL/6J, LG/J, DBA/2J, SJL/J, BALB/cJ, and LP/J. They were housed, one breeding pair per cage, in two cage racks of 36 cages per rack. Each cage rack held six rows of six cages per row. The mice were segregated to one strain per column in the previously listed order. Cages were numbered by column starting with row one.

3.1.2 Mouse Water

Tap water, acidified with five drops of concentrated HCl per 500 mL was available to the mice on an ad lib basis. This level of acidification is recommended as a means of reducing microbiological growth in the water bottles(39).

It also reduces the deposition of calcium and other minerals on the insides of the water bottles. The pH of the water was determined to be 2.58 with a standard deviation of 0.02.

3.1.3 Mouse Diet

Mouse feed of the specific types as listed in Table 3.1.3.1 was available to the mice on an ad lib basis. We note that the DBA/2J mice were very poor breeders when the diet contained more than 6% fat. Other mice such as the BALB/cJ are poor breeders unless the diet contains at least 10% fat. The diets were chosen on the basis of published Jackson Laboratory recommendations, breeding experience in our laboratory, availability, and price (40).

TABLE 3.1.3.1

Diets

	Purina Lab Chow	911A	96WA
C57BL/6J	x	x	
LG/J	x	x	
DBA/2J	x		x

	Purina Lab Chow	911A	96WA
SJL/J		x	
BALB/cJ		x	
LP/J	x	x	

The first row of values in Table 3.1.3.2 are provided by the Ralston Purina Co. These values are typical Purina Lab Chow lot analysis test results. The Old Guilford Lab Diet values for the 911A and 96WA are published Jackson Laboratory specifications (40).

TABLE 3.1.3.2

Diet Composition

Diet	%Fat	%Protein	%Fiber	%Ash
Purina Lab Chow	4.5	23.0	6.0	8.0
911A	10.3	20.1	2.6	4.9
96WA	7.5	22.5	2.3	4.5

3.1.4 Mouse Bedding

Mouse bedding consisted of either autoclaved wood shavings or pelletized and autoclaved sawdust pellets.

3.1.5 Cage Cleaning

Mouse cages and water bottles were washed, cleaned, and recharged with new bedding, water, and food approximately once every ten days.

3.1.6 Mouse Room Environment

The cage racks were located in a working research laboratory on either side of a dual bank of continuously operating laboratory fume hoods. The hoods provided a constant low background noise and a room air change approximately one every three minutes. The laboratory temperature was approximately 20 ^{o}C. The cool white fluorescent lighting was not on timers but was off approximately six to eight hours every night. We were aware of reports of environmental influences on the health of laboratory animals(41). The clean, calm, and consistent laboratory environment resulted in a healthy mouse colony.

3.1.7 Evolving Number System

Sufficient data were maintained on the date of births, cage transfers, age of weaning, pairings, and deaths by the use of weekly cage "Transfer Sheets" to permit the calculation of individual mouse numbers by the evolving number method(42). This system was used during the first year of the colony but was dropped due to the large time requirements for its maintenance with a colony that consisted of 60 breeding pairs and a total population of approximately 300 mice. At each cage cleaning a total count was maintained for all mice by location, sex, strain, and breeding or experimental status.

3.1.8 Mouse Plasma

The following procedure for obtaining mouse plasma was developed as the result of a considerable amount of experimentation with numerous widely different methods and procedures. The procedure described here requires a total collection time of approximately five minutes per mouse for all collection and preparation steps.

One begins by placing the mouse in a "mouse vise" with the tail held outside of the apparatus. It is necessary to firmly hold the end of the tail at all times. The tail is closely shaved with a disposable razor. A prominent tail vein, usually the dorsal vein, is then sliced with a scalpel about 2 cm from the posterior in a direction perpendicular to the long axis of the vein. A 250 mm heparinized haematocrit tube is placed in such a manner that it just touches the drop of blood that forms at the vein cut. The goal is to collect as much blood as possible in the haematocrit tube. The collected whole blood is then blown out of the tube and into a 400 µL microcentrifuge tube. This tube is centrifuged at 4000 rpm for fifteen minutes. The upper plasma layer is removed with a 20 µL Eppendorf micropipet and placed in a 250 µL microcentrifuge tube. This tube, made of conventional polyethylene, is capped and placed in a known location on the grid of a plasma sample board.

Plasma was analyzed rather than serum because experimentation was easier and the difficulty of reproducibly clotting small volumes of whole blood was avoided. The analytical procedure requires at least 50 µL of whole blood prior to the centrifuge step.

This results in at least 25 µL of plasma for analysis thus allowing 10 µL per metal and 5 µL for solution volume. It is rather difficult to consistently and readily obtain such volumes of whole blood from small young mice.

Records of plasma collection were maintained on a "Blood Collection Sheet". These data consist of the mouse cage number, strain, sex, time of collection, date of collection, volume of whole blood collected, and the final grid location on the plasma sample board. The plasma sample board was a rectangular board with a grid network of holes drilled into its surface. Each hole holds a 250 µL conventional polyethylene microcentrifuge tube.

3.2 Miscellaneous Experimental Details

3.2.1 Water

All water used in all experiments was deionized, filtered through a 0.45µ Millipore filter, doubly distilled, and stored in linear polyethylene bottles.

3.2.2 Atomic Absorption Standards

Banco(Anderson Labs, Ft. Worth, Texas) 1000 ppm copper and zinc atomic absorption standards were used in this work. Standards of lower concentration were prepared from these standards by dilution with the previously described water.

3.2.3 Polyethylene Containers

Samples and atomic absorption standards were stored in linear polyethylene containers. The plasma samples were stored in conventional polyethylene microcentrifuge tubes.

3.2.4 Syringes

All flow injections for atomic absorption were done with a 10 µL Hamilton #701 syringe.

3.2.5 Atomic Absorption Lamps

The zinc hollow cathode lamp was from Cathodeon-Sadtler, 3QNY/Zn, Serial #79273. The copper hollow cathode lamp was from Cathodeon-Oriel, #6827-1, Serial #65914.

CHAPTER FOUR

4. Results and Discussion

Procedures for atomic absorption analysis by flow injection were developed for the assay of copper and zinc in 10 µL samples of plasma from age dependent audiogenic seizure susceptible mice. The analysis procedure has a precision of less than 10 %RSD at a copper or zinc concentration or 1.0 ppm. The flow injection apparatus was designed with a pulseless liquid flow control system. Flow regulation is accomplished by varying the inert gas pressure on the pressurized solvent reservoir. The aspiration solvent is 6%(v/v) t-butanol in water. After exhaustive parameter optimizations, copper assays were conducted with the conventional air-acetylene flame at 324.754 nm and zinc assays were conducted with the unconventional argon-hydrogen air diffusion flame at 213.856 nm. The aspiration solvent was chosen because it enhanced the signal without precipitating the plasma proteins. The argon-hydrogen flame system was chosen because it absorbs very little ultraviolet light at the analytical wavelength for zinc (213.856 nm).

The developed methods for atomic absorption by flow injection were used to determine the concentration of copper and zinc in plasma samples from six strains(C57BL/6J, LG/J, DBA/2J, SJL/J, BALB/cJ, and LP/J) of male and female audiogenic seizure susceptible mice, aged 18 to 65 days. The results are presented in 24 sets of data and graphs of metal concentrations versus mouse age.

The data and graphs of plasma copper and zinc concentration versus mouse age for the six strains of mice are presented in the Appendix. A linear least squares reduction of these data is presented in Table and Figure 4.1.1 for copper and 4.2.1 for zinc. For ease of viewing, twelve of these lines are presented on the same graph in Figures 4.1.1 and 4.2.1. An averaged linear least squares reduction of the reduced data is presented in Figure 4.1.2 for copper and 4.2.2 for zinc. This line represents the average copper or zinc concentration versus age for all strains and sexes.

TABLE 4.1.1

Linear Least Squares Calculated Copper Concentrations by Age

#	Strain	Sex	n	Corr.	Slope	Cu, ppm at Age, Days 0	20	40	60
1	C57BL/6J	Male	14	-0.1137	-0.0031	1.0	1.0	0.9	0.8
2	C57BL/6J	Female	14	0.0994	0.0033	0.9	1.0	1.1	1.1
3	LG/J	Male	16	0.3278	0.0098	0.8	1.0	1.2	1.4
4	LG/J	Female	13	0.2905	0.0075	1.0	1.1	1.3	1.4
5	DBA/2J	Male	15	-0.4332	-0.0109	1.5	1.3	1.1	0.8
6	DBA/2J	Female	13	0.1575	0.0037	1.0	1.0	1.1	1.2
7	SJL/J	Male	19	0.2941	0.0065	1.0	1.1	1.2	1.3
8	SJL/J	Female	22	0.1659	0.0040	1.0	1.1	1.2	1.3
9	BALB/cJ	Male	14	-0.1692	-0.0082	1.6	1.4	1.3	1.1
10	BALB/cJ	Female	16	-0.2020	-0.0078	1.7	1.5	1.3	1.2
11	LP/J	Male	10	-0.2062	-0.0052	1.3	1.2	1.1	1.0
12	LP/J	Female	11	-0.1543	-0.0036	1.3	1.2	1.1	1.0

FIGURE 4.1.1

Copper Concentration versus Age for the Plasma of Six Strains of Male and Female Mice

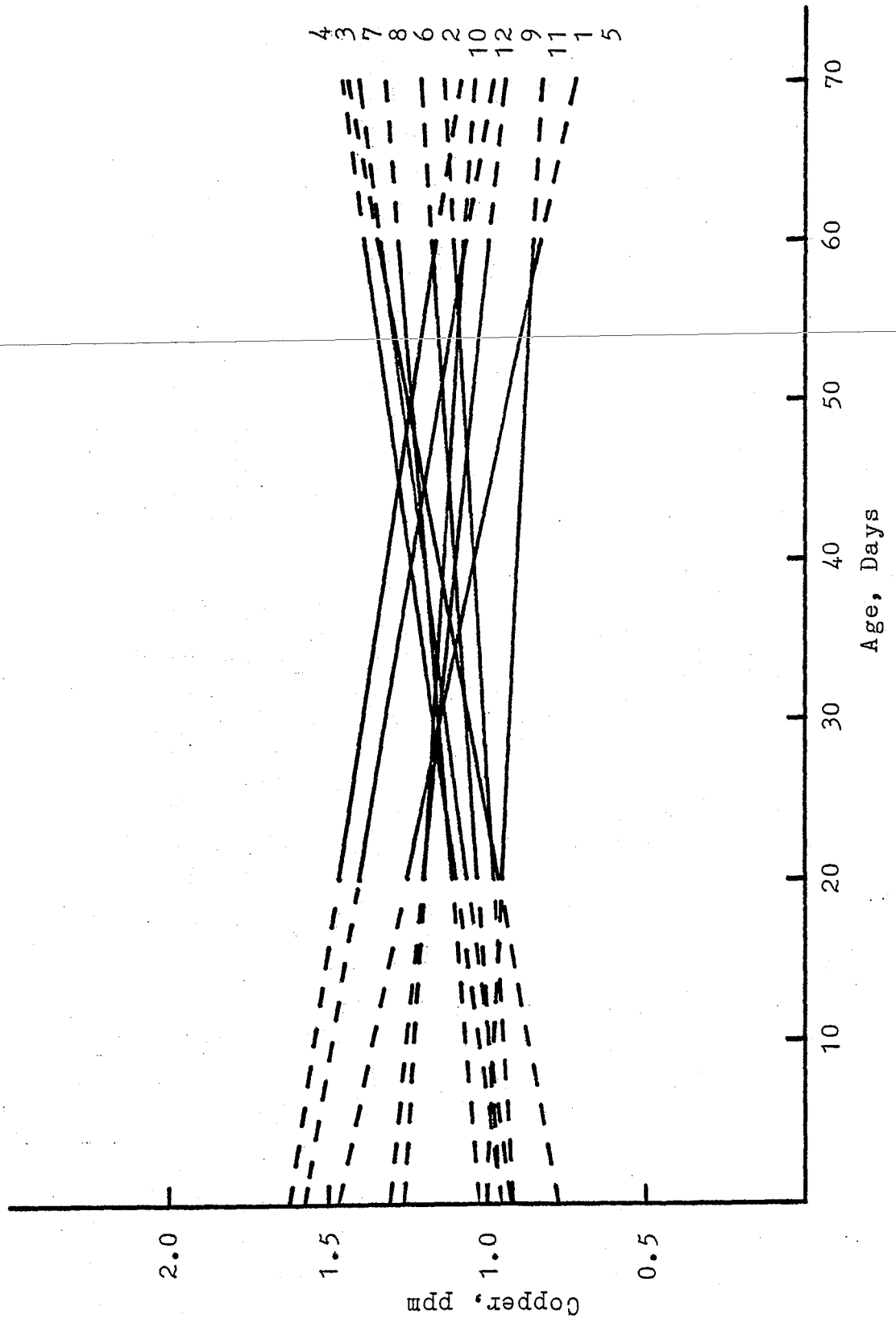

4
3
7
8
6
2
10
12
9
11
1
5
10
20
30
40
50
60
70
Age, Days
2.0
1.5
1.0
0.5
Copper, ppm

FIGURE 4.1.2

Average Copper Concentration versus Age for the Plasma of Six Strains of Male and Female Mice

n	=	4
Correlation	=	-0.8452
Slope	=	-0.0003
x = 0, y =	=	1.16

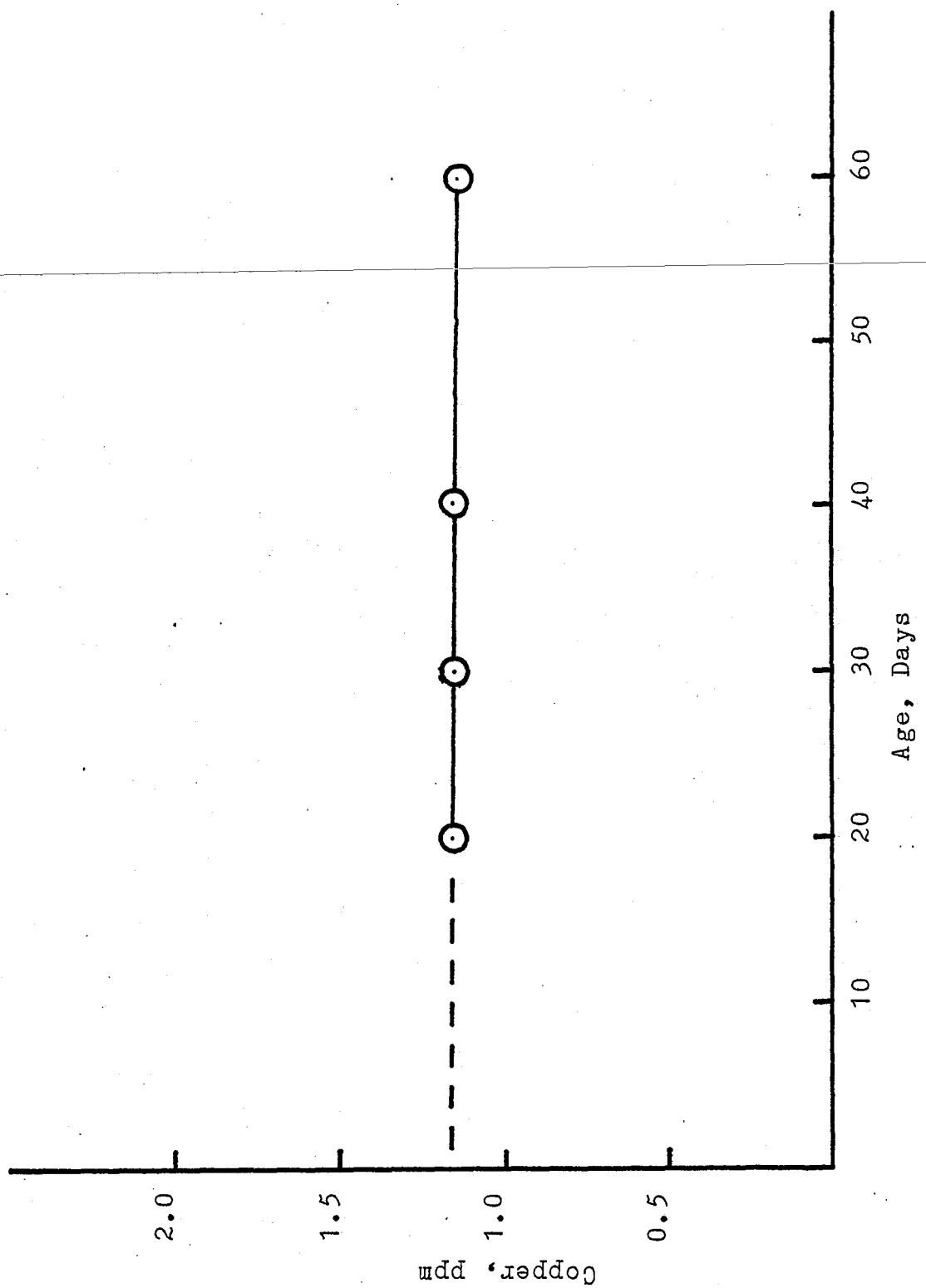

Copper, ppm
2.0
1.5
1.0
0.5
10
20
30
40
50
60
Age, Days

TABLE 4.2.1

Linear Least Squares Calculated Zinc Concentration by Age

#	Strain	Sex	n	Corr.	Slope	Zn, ppm at Age, Days 0	20	40	60
1	C57BL/6J	Male	14	0.6735	0.0242	0.5	1.0	1.4	1.9
2	C57BL/6J	Female	13	0.3982	0.0121	0.9	1.1	1.4	1.6
3	LG/J	Male	16	0.4891	0.0265	0.9	1.4	1.9	2.4
4	LG/J	Female	15	0.5959	0.0147	1.0	1.3	1.6	1.9
5	DBA/2J	Male	13	0.4209	0.0140	0.9	1.2	1.4	1.7
6	DBA/2J	Female	13	0.7826	0.0200	0.6	1.0	1.4	1.8
7	SJL/J	Male	22	0.5170	0.0099	1.2	1.4	1.6	1.8
8	SJL/J	Female	21	0.5557	0.0160	0.9	1.3	1.6	1.9
9	BALB/cJ	Male	12	0.1444	0.0060	1.1	1.2	1.4	1.5
10	BALB/cJ	Female	13	0.2372	0.0057	1.1	1.2	1.3	1.4
11	LP/J	Male	12	0.6482	0.0285	0.3	0.8	1.4	2.0
12	LP/J	Female	11	0.3993	0.0173	0.9	1.2	1.6	1.9

FIGURE 4.2.1

Zinc Concentration versus Age for the Plasma of Six Strains of Male and Female Mice

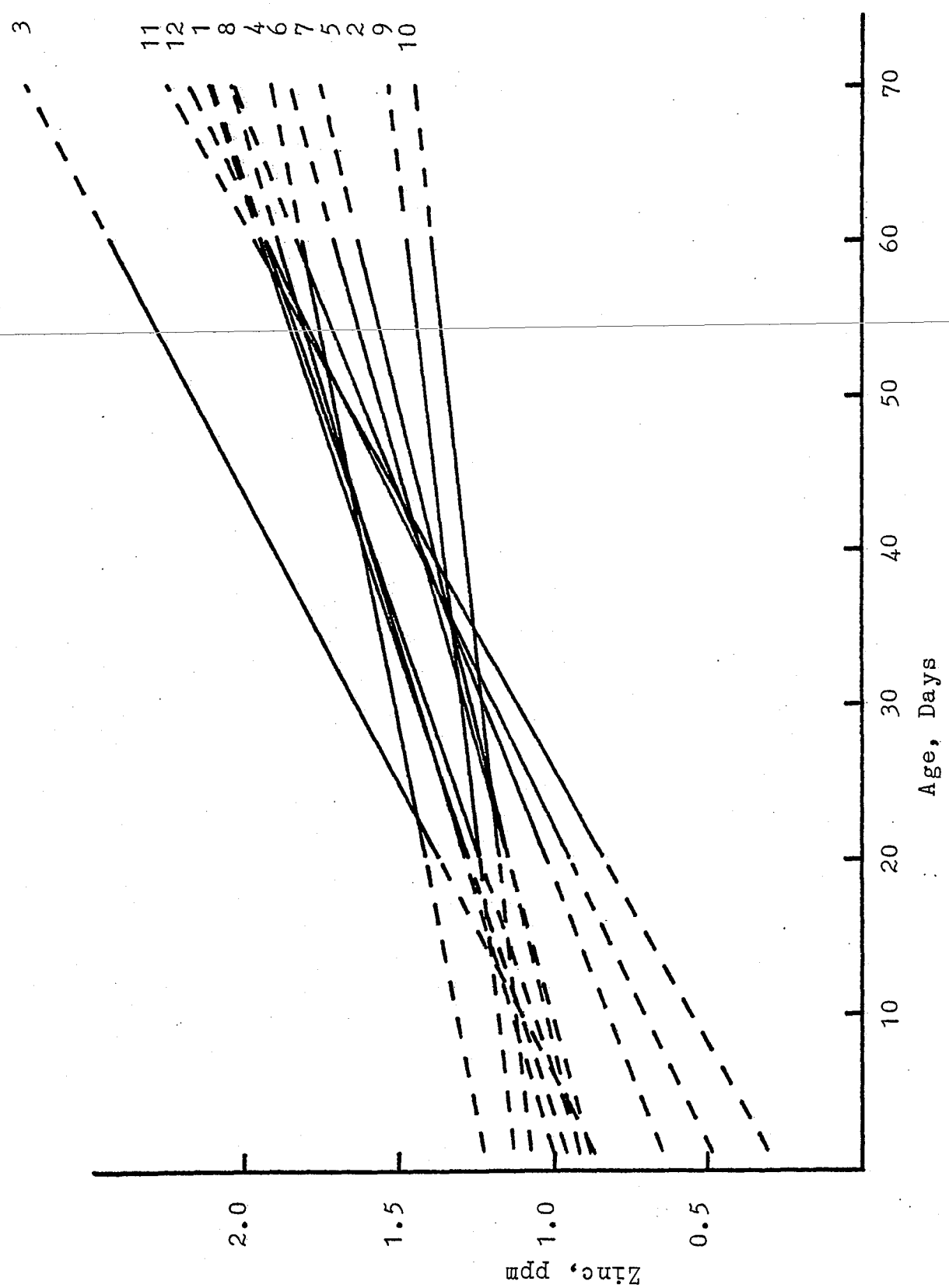
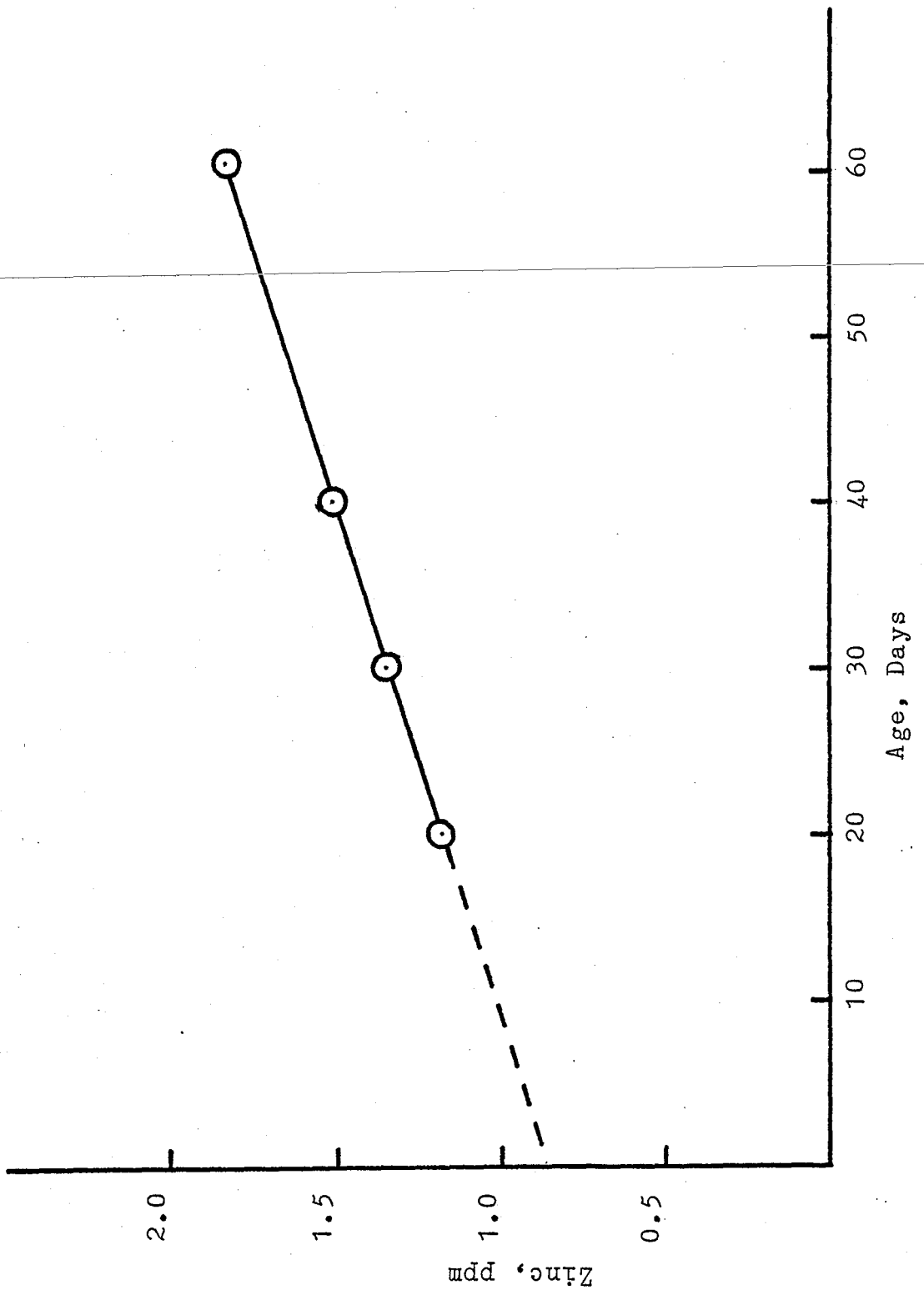

3
11
12
1
8
4
6
7
5
2
9
10
2.0
1.5
1.0
0.5
Zinc, ppm
10
20
30
40
50
60
70
Age, Days

FIGURE 4.2.2

Average Zinc Concentration versus Age for the Plasma of Six Strains of Male and Female Mice

n	=	4
Correlation	=	1.0000
Slope	=	0.0163
x = 0, y	=	0.85


Zinc, ppm
2.0
1.5
1.0
0.5
10
20
30
40
50
60
Age, Days

The copper concentration, within the precision of the data, is 1.2 ppm ± 0.2 SD for all sexes, strains, and ages. The zinc concentration, within the precision of the data, increases from 1.2 ppm ± 0.2 SD at twenty days of age to 1.8 ppm ± 0.2 SD at sixty days of age for all sexes and strains.

There is no correlation between the age dependence of the seizure susceptibility of any sex or strain of mice and their plasma concentrations or concentration ratios for either copper or zinc. If there were a correlation between the copper or zinc plasma concentration and the seizure susceptibility we would expect to observe a significant copper or zinc plasma concentration change that would profile the age dependency of the seizure susceptibility. Such an age dependent concentration profile was not found. The copper and zinc plasma concentration as a function of age does not follow the seizure susceptibility profile as a function of age for any strain or sex of mice studied.

At any age there is no correlation between the relative seizure susceptibility among the strains of mice and their concentrations or concentration ratios of either copper or zinc. If there were a correlation it should follow the interstrain seizure susceptibility pattern of LP/J > DBA/2J > SJL/J > LG/J > BALB/cJ > C57BL/6J.

Such a correlation was not observed.

The age dependence of the percentage audiogenic seizure susceptibility in these strains of mice is very large, ranging from zero to over 97 percent susceptibility. This was shown by the work of Hamburgh and Vicari(33) and later by the work of Fuller and Sjursen(34).

Since a correlation between the age dependence of the audiogenic seizure susceptibility of the mice and their plasma copper or zinc concentration was not found, within the precision of the data, we conclude that such correlations probably do not exist. This result and the lack of any correlation between the relative interstrain seizure susceptibilities and the copper or zinc plasma concentrations suggests the absence of any correlation between copper or zinc plasma concentrations and seizure susceptibility.

Various authors(36,37,38) have reported elevated serum copper concentrations of approximately 1.3 ppm $\pm$ 0.2 SD in epileptic humans receiving medication versus concentrations of approximately 1.1 ppm $\pm$ 0.1 SD in human controls. Olatunbosun has also reported the decrease of serum zinc concentrations from approximately 1.0 ppm $\pm$ 0.1 SD in human controls to approximately 0.8 ppm $\pm$ 0.1 SD in human epileptics receiving medication(37).

It is thus very interesting that we did not find elevated copper and reduced zinc plasma concentrations in our series of inbred strains of age dependent audiogenic seizure susceptible mice.

These results suggest two interesting experiments. The first experiment would be to determine the concentration of copper and zinc in the plasma of human epileptics who had not been receiving anticonvulsant medication. The second experiment would be to determine the age dependence of the copper and zinc plasma concentrations for our series of audiogenic seizure susceptible mice who had been receiving anticonvulsant medication.

These additional experiments would tell whether anticonvulsant medication raises the concentration of copper and lowers the concentration of zinc in the plasma of human epileptics. If it does not, the validity of using audiogenic seizure susceptible mice as a model for human epilepsy is very much in doubt.

CHAPTER FIVE

5. Claims to Originality

1. Sensitivity was enhanced by a factor of 1.7 (Table 1.3.8.1) for atomic absorption flow injection analysis methods by the use of a mixed aqueous-organic solvent, 6%(v/v) t-butanol in water, instead of water.

2. Sensitivity was increased by a factor of 1.25 (Figure 1.3.3.1) for atomic absorption flow injection analysis methods by the use of an argon-hydrogen air diffusion flame instead of an air-acetylene flame.

3. A set of procedures was developed to enable an analyst to increase the precision and sensitivity of the results obtained from atomic absorption flow injection analysis assays by systematically optimizing the instrumentation parameters. The application of these procedures to the analytical system will result in a set of optimal instrumentation parameters.

4. Atomic absorption flow injection analysis methods were applied to the analysis of viscous, salty, aqueous and/or organic, and very complex fluids such as plasma and serum by the use of mixed aqueous-organic solvents to keep such fluids in solution in the solvent stream.

5. Data were obtained for the concentration of copper in the plasma of six strains(C57BL/6J, LG/J, DBA/2J, SJL/J, BALB/cJ, LP/J) of audiogenic seizure susceptible mice, aged 18 to 65 days.

6. Data were obtained for the concentration of zinc in the plasma of six strains (C57BL/6J, LG/J, DBA/2J, SJL/J, BALB/cJ, LP/J) of audiogenic seizure susceptible mice, aged 18 to 65 days.

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APPENDIX

A. Tables and Figures of Plasma Copper and Zinc Concentrations by Age

A.1.1 Tables of Plasma Copper Concentrations by Age

TABLE A.1.1.1.1

Male C57BL/6J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
26	0.9, 0.9	2	0.9	0.0
27	1.2	1	1.2	0.0
28	0.6, 0.5, 0.5, 1.0, 1.1, 1.1, 0.9, 1.1	8	0.9	0.3
29	1.1, 0.7, 0.6, 0.7, 0.6, 0.7	6	0.7	0.2
31	0.9, 0.8, 0.6, 0.7	4	0.8	0.1
32	0.6, 0.7, 1.0, 0.7	4	0.8	0.2
33	0.8, 1.0	2	0.9	0.1
34	1.0, 0.8, 0.8, 0.7, 0.7, 0.9, 1.1, 2.0, 1.1, 1.6, 1.6, 1.0, 1.1, 1.0	14	1.1	0.4
35	1.2, 1.3, 1.2	3	1.2	0.1
37	1.0, 0.9, 0.8, 0.9, 1.2, 1.1, 1.0, 1.5, 0.7	9	1.0	0.2
39	1.0, 0.8, 0.7, 1.1, 0.8, 0.8, 0.9, 0.8	8	0.9	0.1
41	1.1, 0.9, 0.9, 1.0	4	1.0	0.1
43	0.8, 0.9, 1.0, 1.1, 0.7	5	0.9	0.2
44	0.8, 0.6	2	0.7	0.1
		n 14	14	14
		$\bar{x}$ 5	0.9	0.2
		SD 4	0.2	0.1

TABLE A.1.1.1.2

Male LG/J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
25	0.8, 0.7, 0.7, 0.7	4	0.7	0.1
27	1.3, 1.5	2	1.4	0.1
28	0.9, 0.9, 0.7, 1.1, 1.4, 0.9, 1.1	7	1.0	0.2
30	0.8, 0.7, 0.9, 0.7, 1.3, 1.0, 1.5, 1.4, 1.2	9	1.1	0.3
32	0.9, 1.0, 0.8	3	0.9	0.1
34	0.9, 1.2, 1.3, 1.2, 1.1, 1.3, 1.1	7	1.2	0.1
35	1.5, 1.0	2	1.3	0.4
36	1.0, 1.1, 1.0, 1.3, 1.2, 1.8, 1.6, 1.6, 1.3	9	1.3	0.3
38	0.8, 1.0	2	0.9	0.1
39	1.0, 1.1	2	1.1	0.1
40	1.4, 1.6, 0.9	3	1.3	0.4
41	1.3	1	1.3	0.0
42	1.3, 1.2, 1.0, 1.2	4	1.2	0.1
44	0.7, 0.8, 1.0, 1.0, 0.8	5	0.9	0.1
48	1.4, 1.6	2	1.5	0.1
49	1.1, 1.1	2	1.1	0.0
	$\underline{n}$	16	16	16
	$\bar{x}$	4	1.1	0.2
	SD	3	0.2	0.1

TABLE A.1.1.1.3

Male DBA/2J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
26	1.4	1	1.4	0.0
27	1.2, 1.1	2	1.2	0.1
32	0.9, 1.0, 1.0	3	1.0	0.1
33	1.5, 1.2	2	1.4	0.2
34	0.7	1	0.7	0.0
35	1.2, 1.2	2	1.2	0.0
36	1.2, 1.1, 1.0, 1.1, 1.0	5	1.1	0.1
38	1.0, 1.1, 1.3	3	1.1	0.2
40	0.9, 1.2, 0.9, 1.2, 1.4	5	1.1	0.2
41	0.9	1	0.9	0.0
44	0.9, 1.0, 1.1	3	1.0	0.1
45	0.9	1	0.9	0.0
47	0.8, 0.9, 1.1	3	0.9	0.2
49	0.9	1	0.9	0.0
52	1.2	1	1.2	0.0
	n	15	15	15
	$\bar{x}$	2	1.1	0.1
	SD	1	0.2	0.1

TABLE A.1.1.1.4

Male SJL/J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
18	1.1	1	1.1	0.0
19	1.2, 1.3, 1.3	3	1.3	0.1
25	1.1, 1.2, 1.3, 1.5, 1.4	5	1.3	0.2
26	1.1, 1.1, 1.2	3	1.1	0.1
29	1.3, 1.0, 1.3	3	1.2	0.2
30	1.2, 1.1	2	1.2	0.1
32	1.2, 1.2, 0.9, 1.2, 1.1	5	1.1	0.1
35	0.9, 0.8, 0.9, 0.9, 0.8, 0.6	6	0.8	0.1
36	1.3, 1.0	2	1.2	0.2
37	1.1, 1.1, 1.0, 1.0, 1.0	5	1.0	0.1
38	1.4, 1.0, 1.2, 1.2, 0.7, 0.6, 0.8	7	1.0	0.3
40	1.0, 1.0	2	1.0	0.0
41	1.0, 0.7, 0.9, 1.0, 0.9	5	0.9	0.1
42	0.9, 1.1, 1.0, 1.1, 1.6, 1.4, 1.5, 1.6, 1.0	9	1.2	0.3
44	0.9, 0.6, 0.9, 1.0	4	0.9	0.2
49	1.5, 1.8	2	1.7	0.2
50	1.2, 2.0	2	1.6	0.6
52	1.5, 1.1, 1.8, 1.0	4	1.4	0.4
53	1.3	1	1.3	0.0
	n	19	19	19
	$\bar{x}$	4	1.2	0.2
	SD	2	0.2	0.1

TABLE A.1.1.1.5

Male BALB/cJ Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
21	1.3, 1.8, 1.0, 1.3	4	1.4	0.3
24	1.5, 0.8, 0.8, 0.8	4	1.0	0.4
25	1.3, 2.1, 1.6, 1.3	4	1.6	0.4
28	1.7, 2.4	2	2.1	0.5
29	1.4, 1.5	2	1.5	0.1
31	1.1, 0.8	2	1.0	0.2
32	1.1	1	1.1	0.0
35	1.6, 1.4, 1.0, 1.2, 1.0, 0.8, 0.7	7	1.1	0.3
37	1.3	1	1.3	0.0
39	0.7, 0.7	2	0.7	0.0
40	1.4	1	1.4	0.0
41	1.4	1	1.4	0.0
43	1.8	1	1.8	0.0
45	1.0	1	1.0	0.0
	n	14	14	14
	$\bar{x}$	2	1.3	0.2
	SD	2	0.4	0.2

TABLE A.1.1.1.6

Male LP/J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
22	1.4	1	1.4	0.0
27	0.9, 0.8	2	0.9	0.1
28	1.2, 1.2	2	1.2	0.0
34	1.1, 1.3	2	1.2	0.1
37	1.1, 0.8	2	1.0	0.2
41	1.7, 1.5, 1.5	3	1.6	0.1
45	0.6	1	0.6	0.0
49	1.0	1	1.0	0.0
50	1.0, 1.2	2	1.1	0.1
54	1.3, 1.1	2	1.2	0.1
		10	10	10
	$\underline{n}$	2	1.1	0.1
	$\bar{x}$	1	0.3	0.1
	SD			

TABLE A.1.1.2.1

Female C57BL/6J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
27	0.8, 0.5, 2.7, 1.5	4	1.4	1.0
28	0.6, 0.7, 0.5, 0.6, 0.5, 0.9, 1.1, 1.0, 1.0, 1.2	10	0.8	0.3
29	0.7, 1.0	2	0.9	0.2
31	0.8	1	0.8	0.0
32	0.8, 0.7, 0.5	3	0.7	0.2
33	0.8, 0.7, 0.8, 0.8, 1.4, 1.4, 1.3	7	1.0	0.3
34	1.3, 1.2	2	1.3	0.1
35	1.1	1	1.1	0.0
37	1.1, 1.3, 1.3, 1.1, 1.3, 1.1, 1.3, 1.5, 0.6, 1.0, 1.1	11	1.2	0.2
39	1.5, 0.8, 1.0	3	1.1	0.4
41	1.5, 0.9, 0.9	3	1.1	0.3
43	1.0, 1.6	2	1.3	0.4
44	0.8, 0.8	2	0.8	0.0
48	1.0, 0.9	2	1.0	0.1
	n	14	14	14
	$\bar{x}$	4	1.0	0.3
	SD	3	0.2	0.3

TABLE A.1.1.2.2

Female LG/J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
27	1.5	1	1.5	0.0
28	0.9, 1.3, 1.2, 1.5, 1.2, 1.1, 1.0, 1.2, 1.4	9	1.2	0.2
32	0.7, 0.8	2	0.8	0.1
34	1.5, 1.5, 1.1, 1.4	4	1.4	0.2
36	1.1, 1.4, 1.2, 1.2, 1.1, 1.2	6	1.2	0.1
38	0.9, 0.9	2	0.9	0.0
40	1.0, 0.6, 1.3	3	1.0	0.4
41	1.6	1	1.6	0.0
42	1.7, 1.0	2	1.4	0.5
44	0.8, 1.0, 1.3, 0.8	4	1.0	0.2
48	1.6	1	1.6	0.0
61	1.7	1	1.7	0.0
64	1.2	1	1.2	0.0
	n	13	13	13
	$\bar{x}$	3	1.3	0.1
	SD	2	0.3	0.2

TABLE A.1.1.2.3

Female DBA/2J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
27	1.1, 1.2, 1.2	3	1.2	0.1
32	1.2, 0.9, 1.1	3	1.1	0.2
34	0.8, 0.8	2	0.8	0.0
35	1.2, 1.1, 1.0, 1.5	4	1.2	0.2
36	1.0, 1.2	2	1.1	0.1
38	1.1, 1.4, 1.2	3	1.2	0.2
40	1.3, 1.3, 0.9, 0.9, 1.3, 1.0	6	1.1	0.2
41	1.2, 1.1	2	1.2	0.1
45	0.8, 0.7, 0.6	3	0.7	0.1
49	1.0, 1.1	2	1.1	0.1
50	1.1, 1.6, 1.7	3	1.5	0.3
52	1.1, 1.2, 1.0	3	1.1	0.1
54	1.2	1	1.2	0.0
	n	13	13	13
	$\bar{x}$	3	1.1	0.1
	SD	1	0.2	0.1

TABLE A.1.1.2.4

Female SJL/J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
18	1.1, 0.8, 0.8, 1.1, 0.9	5	0.9	0.2
19	1.2	1	1.2	0.0
23	1.1, 1.0, 0.8, 1.0	4	1.0	0.1
25	1.2	1	1.2	0.0
26	1.7	1	1.7	0.0
29	1.0, 1.4, 1.2	3	1.2	0.2
30	1.2, 1.3	2	1.3	0.1
32	1.3, 1.2, 1.3, 1.2	4	1.3	0.1
35	1.0, 1.0, 1.0, 1.2, 1.2, 0.7, 0.7, 0.8, 0.8, 0.8	10	0.9	0.2
36	1.2, 1.0	2	1.1	0.1
37	1.1, 1.0, 1.1, 0.9, 1.1, 0.9	6	1.0	0.1
38	1.3, 1.3, 0.7, 0.6, 0.6, 0.8, 0.7, 0.6	8	0.8	0.3
39	1.7, 1.3	2	1.5	0.3
40	0.9, 0.9, 0.9, 1.1, 1.6	5	1.1	0.3
41	0.9, 0.8, 1.0, 0.8, 0.9	5	0.9	0.1
42	1.1, 1.2, 1.2, 1.5, 2.0, 1.3	6	1.4	0.3
44	0.7, 0.7, 0.8, 0.9, 0.9, 0.8, 1.9, 1.6, 1.6	9	1.1	0.5
45	1.2	1	1.2	0.0
49	1.1, 1.1, 1.3, 0.9, 1.0	5	1.1	0.1
50	1.1, 1.9	2	1.5	0.6
52	1.0	1	1.0	0.0
53	0.9, 2.4, 2.2, 1.3	4	1.7	0.7
	n	22	22	22
	$\bar{x}$	4	1.2	0.2
	SD	3	0.3	0.2

TABLE A.1.1.2.5

Female BALB/cJ Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
21	1.5, 1.3	2	1.4	0.1
25	1.5, 1.5, 1.4, 1.3	4	1.4	0.1
28	1.9, 1.7, 1.3, 1.6, 1.5	5	1.6	0.2
29	1.6, 2.1, 1.3	3	1.7	0.4
30	1.0, 1.2	2	1.1	0.1
31	1.0, 1.0	2	1.0	0.0
32	1.7, 1.8, 1.4, 1.0, 1.0	5	1.4	0.4
35	1.0, 1.3, 1.0, 0.8, 0.8	5	1.0	0.2
37	1.6	1	1.6	0.0
40	1.0, 2.0, 2.1	3	1.7	0.6
41	1.8	1	1.8	0.0
42	1.0, 0.9, 1.0, 1.0, 0.9	5	1.0	0.1
43	2.1	1	2.1	0.0
45	1.0, 1.0, 1.1	3	1.0	0.1
51	0.9, 0.8, 1.4	3	1.0	0.3
54	1.0	1	1.0	0.0
		16	16	16
	$\bar{n}$	3	1.4	0.2
	SD	2	0.4	0.2

TABLE A.1.1.2.6

Female LP/J Plasma Copper Concentrations by Age

Age, Days	Cu, ppm	n	$\bar{x}$	SD
22	1.0	1	1.0	0.0
26	1.4	1	1.4	0.0
32	0.8	1	0.8	0.0
34	1.2, 1.1	2	1.2	0.1
36	1.2	1	1.2	0.0
37	1.8, 1.2, 0.8, 0.8	4	1.2	0.5
41	1.6	1	1.6	0.0
43	1.0, 1.3	2	1.2	0.2
44	1.1, 1.1	2	1.1	0.0
50	1.0	1	1.0	0.0
54	0.9	1	0.9	0.0
		11	11	11
	$\bar{n}$	2	1.1	0.1
	$\bar{x}$	1	0.2	0.2
	SD			

A.1.2 Tables of Plasma Zinc Concentrations by Age

TABLE A.1.2.1.1

Male C57BL/6J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
23	0.7, 1.0	2	0.9	0.2
26	1.0, 1.0, 1.2	3	1.1	0.1
27	1.4	1	1.4	0.0
28	1.4, 1.1, 0.9, 0.8, 1.1, 1.4	6	1.1	0.2
29	1.4, 1.0	2	1.2	0.3
31	1.0, 1.1, 1.0, 1.6, 1.3	5	1.2	0.3
32	0.8, 1.3, 1.3, 1.0	4	1.1	0.2
34	1.2, 1.1, 1.0, 1.0, 1.1, 1.4, 1.3, 1.2, 1.3	9	1.2	0.1
35	2.0, 1.1, 2.0	3	1.7	0.5
37	1.3, 1.3, 1.1, 1.2, 1.5, 1.1, 1.5, 1.7, 1.8, 1.0, 1.7, 0.7, 0.9, 1.4	14	1.3	0.3
39	0.9, 1.0, 1.2, 1.1, 1.3, 1.2, 1.3, 1.5, 0.8, 1.0	10	1.1	0.2
41	1.8, 1.4, 1.4, 1.6, 1.4, 1.8, 1.8, 1.3, 1.5	9	1.6	0.2
43	1.6, 1.4, 1.5, 2.0, 1.6	5	1.6	0.2
44	2.0, 1.6, 1.6, 1.3, 1.2	5	1.5	0.3
	n	14	14	14
	$\bar{x}$	6	1.3	0.2
	SD	4	0.2	0.1

TABLE A.1.2.1.2

Male LG/J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
23	1.6, 1.2, 0.9	3	1.2	0.4
25	2.2, 2.0, 1.9, 2.3	4	2.1	0.2
27	1.4, 1.6	2	1.5	0.1
28	1.5, 1.3, 1.9, 1.1	4	1.5	0.3
30	1.8, 1.8, 2.0, 1.6, 1.3, 1.2, 0.6	7	1.5	0.5
32	2.1, 1.2, 1.6	3	1.6	0.5
34	2.0, 2.7, 2.4, 2.4, 1.9, 1.5, 1.4, 1.6	8	2.0	0.5
35	2.4, 2.0, 1.5	3	2.0	0.5
36	1.9, 2.3, 2.4, 1.5, 1.6, 1.6, 1.2, 1.4	8	1.7	0.4
38	2.0, 1.7	2	1.9	0.2
39	2.5, 2.2	2	2.4	0.2
40	0.7, 1.4, 0.8	3	1.0	0.4
42	2.4, 1.8, 1.5, 1.1	4	1.7	0.5
44	2.2, 2.2, 1.6, 2.0, 2.0, 1.5	6	1.9	0.3
48	2.0	1	2.0	0.0
49	1.8, 3.6	2	2.7	1.3
	n	16	16	16
	$\bar{x}$	4	1.8	0.4
	SD	2	0.4	0.3

TABLE A.1.2.1.3

Male DBA/2J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
24	1.3, 1.6	2	1.5	0.2
26	1.4	1	1.4	0.0
27	1.1, 1.4, 1.0	3	1.2	0.2
32	1.0	1	1.0	0.0
35	0.9, 1.3, 1.3	3	1.2	0.2
36	1.9, 2.0, 1.4, 1.4, 1.7	5	1.7	0.3
38	0.5, 1.0, 1.2	3	0.9	0.4
40	1.3, 1.3, 1.6, 1.3, 1.6, 1.3, 1.9, 2.3, 2.0, 1.8	10	1.6	0.4
41	1.1	1	1.1	0.0
44	1.6, 1.3	2	1.5	0.2
47	1.7, 1.9, 1.4, 1.4	4	1.6	0.2
49	1.9	1	1.9	0.0
52	1.6	1	1.6	0.0
		n 13	13	13
		$\bar{x}$ 3	1.4	0.2
		SD 3	0.3	0.2

TABLE A.1.2.1.4

Male SJL/J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
18	1.7	1	1.7	0.0
19	1.4, 1.1	2	1.3	0.2
23	1.4	1	1.4	0.0
25	1.4, 1.3	2	1.4	0.1
26	1.2, 2.2	2	1.7	0.7
29	1.6, 1.1	2	1.4	0.4
30	1.9, 1.3, 1.5, 1.1, 0.9	5	1.3	0.4
31	1.3, 1.3, 1.6, 1.6	4	1.5	0.2
32	1.6, 1.8	2	1.7	0.1
35	1.4, 1.3, 1.4	3	1.4	0.1
36	1.1, 1.4	2	1.3	0.2
37	1.6, 1.5, 1.4	3	1.5	0.1
38	1.8, 1.9, 1.6, 1.5	4	1.7	0.2
40	1.8, 1.2	2	1.5	0.4
41	2.0, 1.8, 1.6, 1.5, 1.6, 1.4	6	1.7	0.2
42	0.9, 1.7, 1.4, 2.0, 1.2	5	1.4	0.4
44	1.7, 2.1, 1.6	3	1.8	0.3
45	1.9	1	1.9	0.0
49	1.2, 2.1, 1.5, 1.6	4	1.6	0.4
50	1.5, 1.7	2	1.6	0.1
52	1.8, 1.1, 1.9, 2.1, 1.9, 1.6	6	1.7	0.4
53	1.7, 2.3	2	2.0	0.4
	$\bar{n}$	22	22	22
	$\bar{\bar{x}}$	3	1.6	0.2
	SD	2	0.2	0.2

TABLE A.1.2.1.5

Male BALB/cJ Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
21	0.6, 0.9	2	0.8	0.2
24	2.0, 1.4	2	1.7	0.4
25	1.2, 1.1, 1.1	3	1.1	0.1
28	1.0, 1.3	2	1.2	0.2
29	1.0, 1.4	2	1.2	0.2
30	1.6	1	1.6	0.0
32	1.5	1	1.5	0.0
33	1.8, 1.6	2	1.7	0.1
35	1.2, 1.1, 0.9, 1.2	4	1.1	0.1
37	1.3, 1.7	2	1.5	0.3
41	0.7, 1.2	2	1.0	0.4
45	1.5, 1.2	2	1.4	0.2
	n	12	12	12
	$\bar{x}$	2	1.3	0.2
	SD	1	0.3	0.1

TABLE A.1.2.1.6

Male LP/J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
22	0.4	1	0.4	0.0
27	1.2, 1.3	2	1.3	0.1
28	1.5	1	1.5	0.0
32	1.2	1	1.2	0.0
34	1.1	1	1.1	0.0
37	1.2, 1.4, 1.1	3	1.2	0.2
39	1.7	1	1.7	0.0
41	1.4, 1.3	2	1.4	0.1
42	1.2	1	1.2	0.0
43	1.9, 1.4, 1.3	3	1.5	0.3
49	1.3	1	1.3	0.0
50	2.0	1	2.0	0.0
		12	12	12
	$\bar{n}$	2	1.3	0.1
	$\bar{x}$	1	0.4	0.1
	SD			

TABLE A.1.2.2.1

Female C57BL/6J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
23	0.9, 1.0, 1.4	3	1.1	0.3
28	1.3, 1.2, 1.4, 1.3, 1.3, 1.6, 1.3, 1.3	8	1.3	0.1
29	1.4	1	1.4	0.0
31	1.4, 1.5, 1.1	3	1.3	0.2
32	1.0, 1.2, 1.2	3	1.1	0.1
33	1.4, 1.7, 0.9	3	1.3	0.4
34	1.3, 1.5	2	1.4	0.1
35	1.2, 1.6	2	1.4	0.3
37	1.4, 1.5, 1.8, 1.7, 1.6, 1.8, 1.4, 1.4, 1.5, 1.3, 1.2, 1.3, 1.9, 1.7, 1.5, 0.9	16	1.5	0.3
39	1.2, 1.1, 1.3	3	1.2	0.1
41	1.4, 1.4, 0.9	3	1.2	0.3
43	1.3, 1.1	2	1.2	0.1
44	2.0, 1.7, 1.6	3	1.8	0.2
	n	13	13	13
	$\bar{x}$	4	1.3	0.2
	SD	4	0.2	0.1

TABLE A.1.2.2.2

Female LG/J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
23	1.6, 1.5	2	1.6	0.1
27	1.4	1	1.4	0.0
28	1.2, 1.5, 1.4, 1.3	4	1.4	0.1
30	1.2	1	1.2	0.0
32	1.4, 1.5, 1.2	3	1.4	0.2
34	0.9, 0.8, 1.8	3	1.2	0.6
36	1.4, 1.0, 1.2, 1.2, 1.5	5	1.3	0.2
38	1.8, 1.8	2	1.8	0.0
39	2.0	1	2.0	0.0
40	1.7, 1.7, 1.9	3	1.8	0.1
42	2.0, 1.4, 1.1	3	1.5	0.5
44	1.8, 1.8, 1.9, 1.3	4	1.7	0.3
49	1.4	1	1.4	0.0
60	1.9	1	1.9	0.0
61	2.1, 1.8, 2.2	3	2.0	0.2
		15	15	15
	$\underline{n}$	15	15	15
	$\bar{x}$	3	1.6	0.2
	SD	1	0.3	0.2

TABLE A.1.2.2.3

Female DBA/2J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
24	1.3, 1.3	2	1.3	0.0
27	1.0, 1.1, 1.1	3	1.1	0.1
32	1.4	1	1.4	0.0
35	1.0, 1.2, 1.0	3	1.1	0.1
36	1.7, 1.2	2	1.5	0.4
38	1.3	1	1.3	0.0
40	1.0, 1.2, 1.4, 1.1, 1.3, 1.6, 1.6	7	1.3	0.2
41	1.1, 1.1, 1.5	3	1.2	0.2
48	1.9, 1.5	2	1.7	0.3
49	1.3, 1.5, 1.7	3	1.5	0.2
50	1.6	1	1.6	0.0
52	1.7, 1.6, 1.8	3	1.7	0.1
54	1.5, 2.2	2	1.9	0.5
	$\bar{n}$	13	13	13
	$\bar{\bar{x}}$	3	1.4	0.2
	SD	2	0.2	0.2

TABLE A.1.2.2.4

Female SJL/J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
18	1.3, 1.3, 1.2, 1.4, 1.4	5	1.3	0.1
23	1.1, 1.1, 1.0, 1.4	4	1.2	0.2
25	1.9	1	1.9	0.0
26	1.3	1	1.3	0.0
29	1.0, 1.4	2	1.2	0.3
30	1.5, 2.2, 1.0, 1.0, 1.1	5	1.4	0.5
31	1.2, 1.5, 1.6, 1.4, 1.4, 1.4	6	1.4	0.1
35	2.0, 1.4, 1.4, 1.7, 1.8, 1.6, 2.2	7	1.7	0.3
36	1.0, 1.4	2	1.2	0.3
37	1.8, 1.2, 1.3, 1.2	4	1.4	0.3
38	1.5, 1.6, 1.3, 1.1, 1.2, 1.1	6	1.3	0.2
39	1.9, 1.9, 1.7, 1.4	4	1.7	0.2
40	1.7, 2.0, 1.6, 1.8	4	1.8	0.2
41	1.6, 1.2, 1.7, 1.9, 1.6	5	1.6	0.3
42	1.6, 1.0, 1.2, 1.7, 0.4	5	1.2	0.5
44	1.6, 2.0, 1.5, 1.7, 1.9, 0.8, 2.0	7	1.6	0.4
45	1.5, 1.9, 2.0, 2.1	4	1.9	0.3
49	1.7, 1.7, 1.5, 1.7, 1.8	5	1.7	0.1
50	1.8, 1.7	2	1.8	0.1
52	2.2	1	2.2	0.0
53	1.9, 1.3, 1.5, 1.6	4	1.6	0.3
	$\underline{n}$	21	21	21
	$\bar{\bar{x}}$	4	1.5	0.2
	SD	2	0.3	0.1

TABLE A.1.2.2.5

Female BALB/cJ Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
21	1.2, 0.9	2	1.1	0.2
25	0.8, 1.4, 1.1	3	1.1	0.3
28	1.5, 1.4, 1.8, 1.9	4	1.7	0.2
29	1.1, 1.6	2	1.4	0.4
30	0.9, 0.7	2	0.8	0.1
32	1.3, 1.4, 1.2, 1.3	4	1.3	0.1
35	2.1, 0.7, 1.0, 1.4, 1.7	5	1.4	0.6
41	0.6, 1.1	2	0.9	0.4
42	1.3, 1.3, 1.4, 1.4, 1.4, 1.4	6	1.4	0.1
43	1.1, 1.2	2	1.2	0.1
45	1.1, 1.4, 1.2, 1.3	4	1.3	0.1
51	1.5, 1.3, 1.6, 1.8, 1.4	5	1.5	0.2
54	1.4, 1.6, 1.5, 1.2	4	1.4	0.2
	$\underline{n}$	13	13	13
	$\bar{x}$	4	1.3	0.2
	SD	1	0.2	0.2

TABLE A.1.2.2.6

Female LP/J Plasma Zinc Concentrations by Age

Age, Days	Zn, ppm	n	$\bar{x}$	SD
22	1.0	1	1.0	0.0
26	1.5	1	1.5	0.0
32	1.8	1	1.8	0.0
34	1.7	1	1.7	0.0
37	1.0, 1.0, 0.7, 1.2	4	1.0	0.2
39	1.5	1	1.5	0.0
41	1.7	1	1.7	0.0
42	2.3	1	2.3	0.0
43	1.0, 1.1, 1.0, 1.4	4	1.1	0.2
50	1.5	1	1.5	0.0
54	2.0	1	2.0	0.0
		11	11	11
	$\bar{n}$	2	1.6	0.0
	$\bar{x}$	1	0.4	0.1
	SD			

A.1.3 Figures of Plasma Copper Concentrations versus Age

FIGURE A.1.3.1.1

Male Plasma Copper Concentrations
versus Age for C57BL/6J Mice

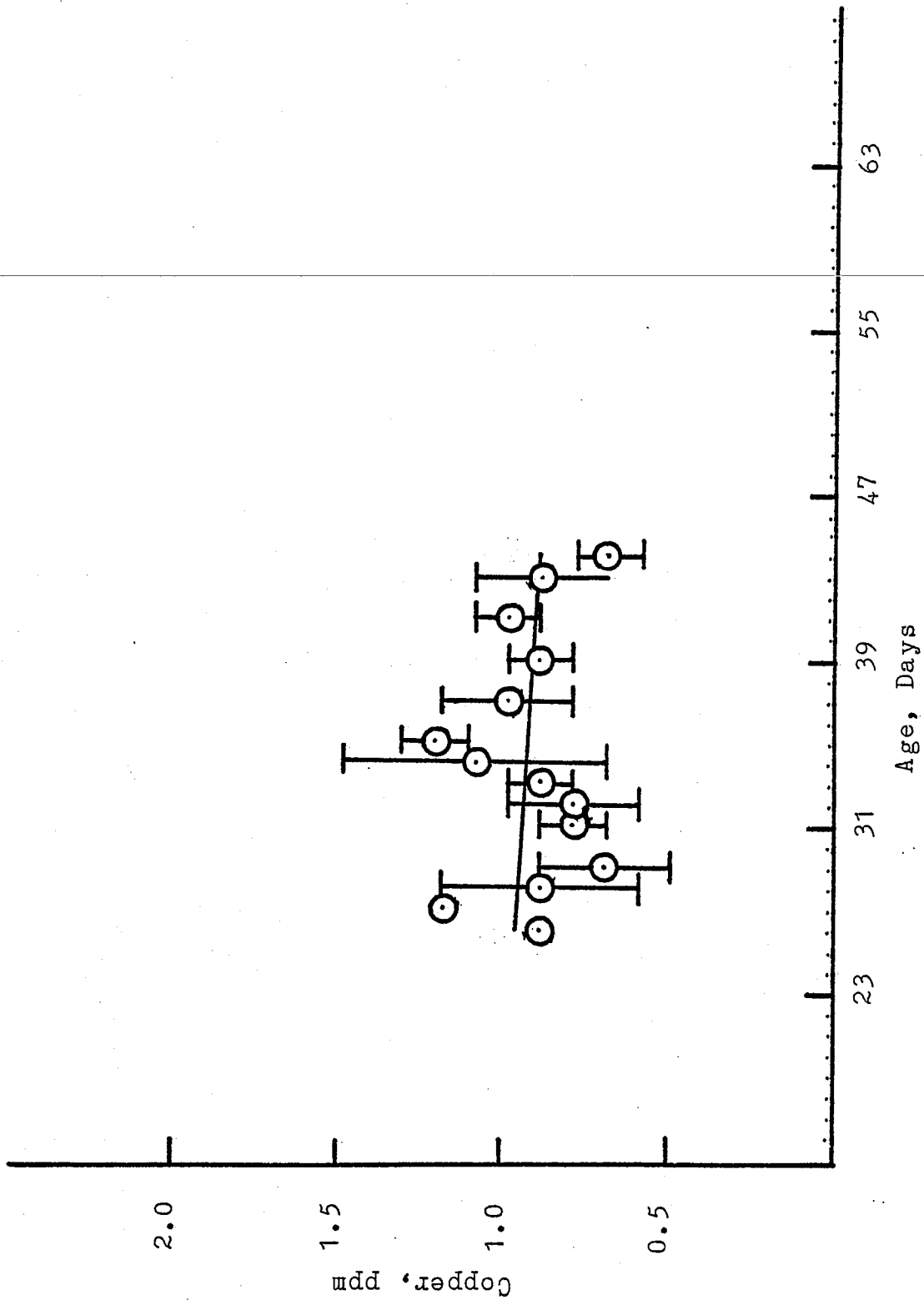

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.1.2

Male Plasma Copper Concentrations
versus Age for LG/J Mice

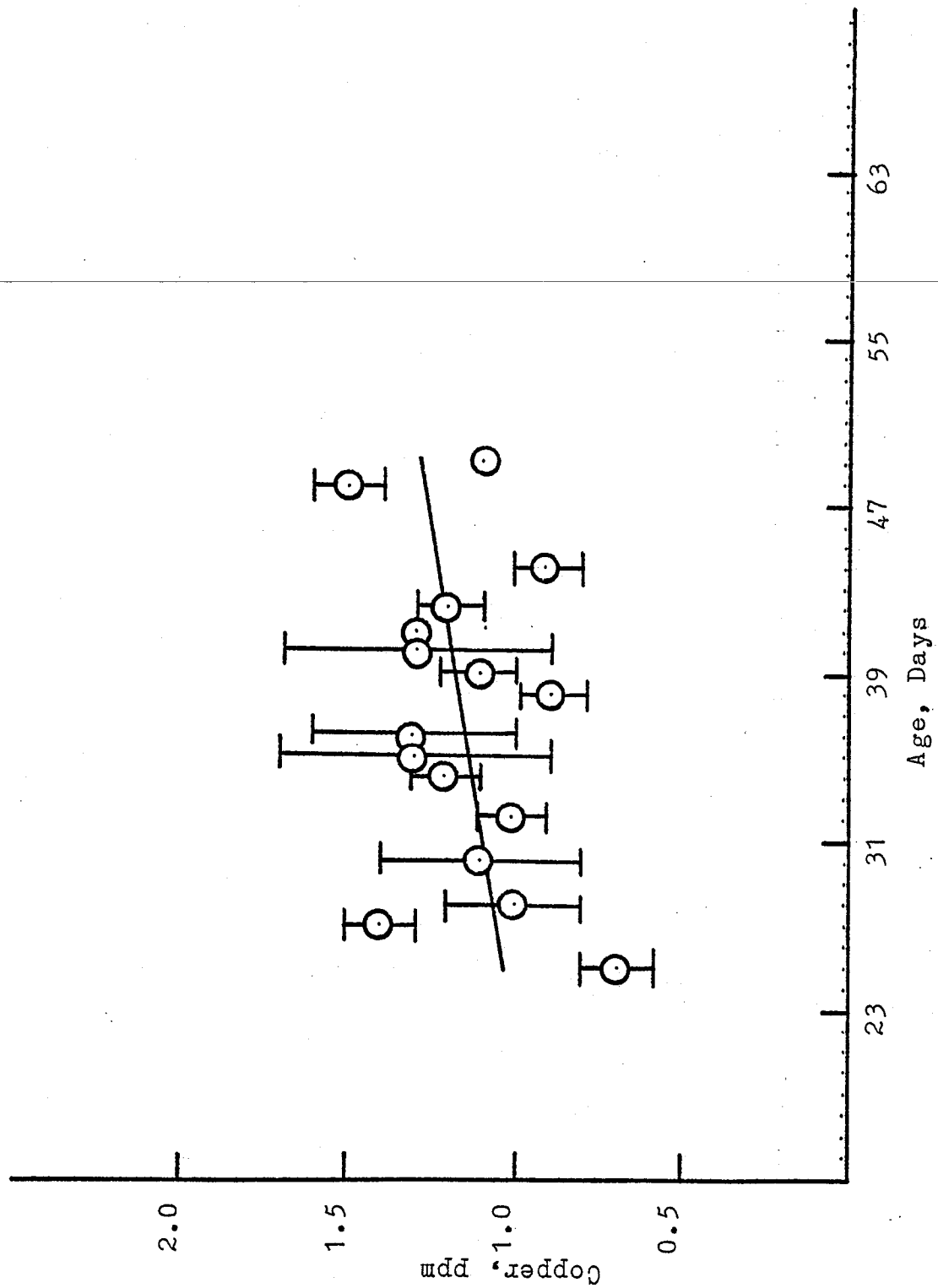

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.1.3

Male Plasma Copper Concentrations
versus Age for DBA/2J Mice

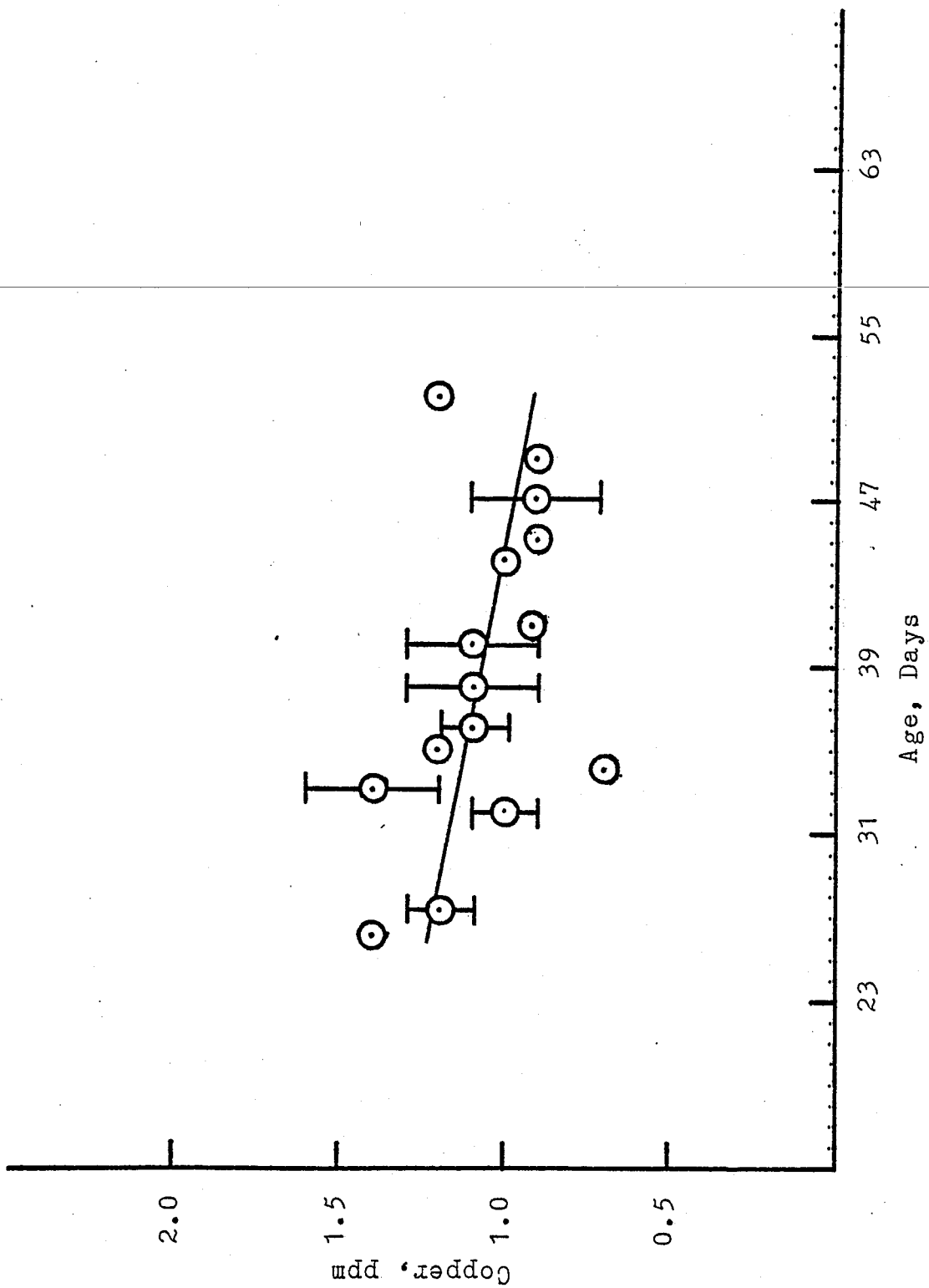

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.1.4

Male Plasma Copper Concentrations
versus Age for SJL/J Mice

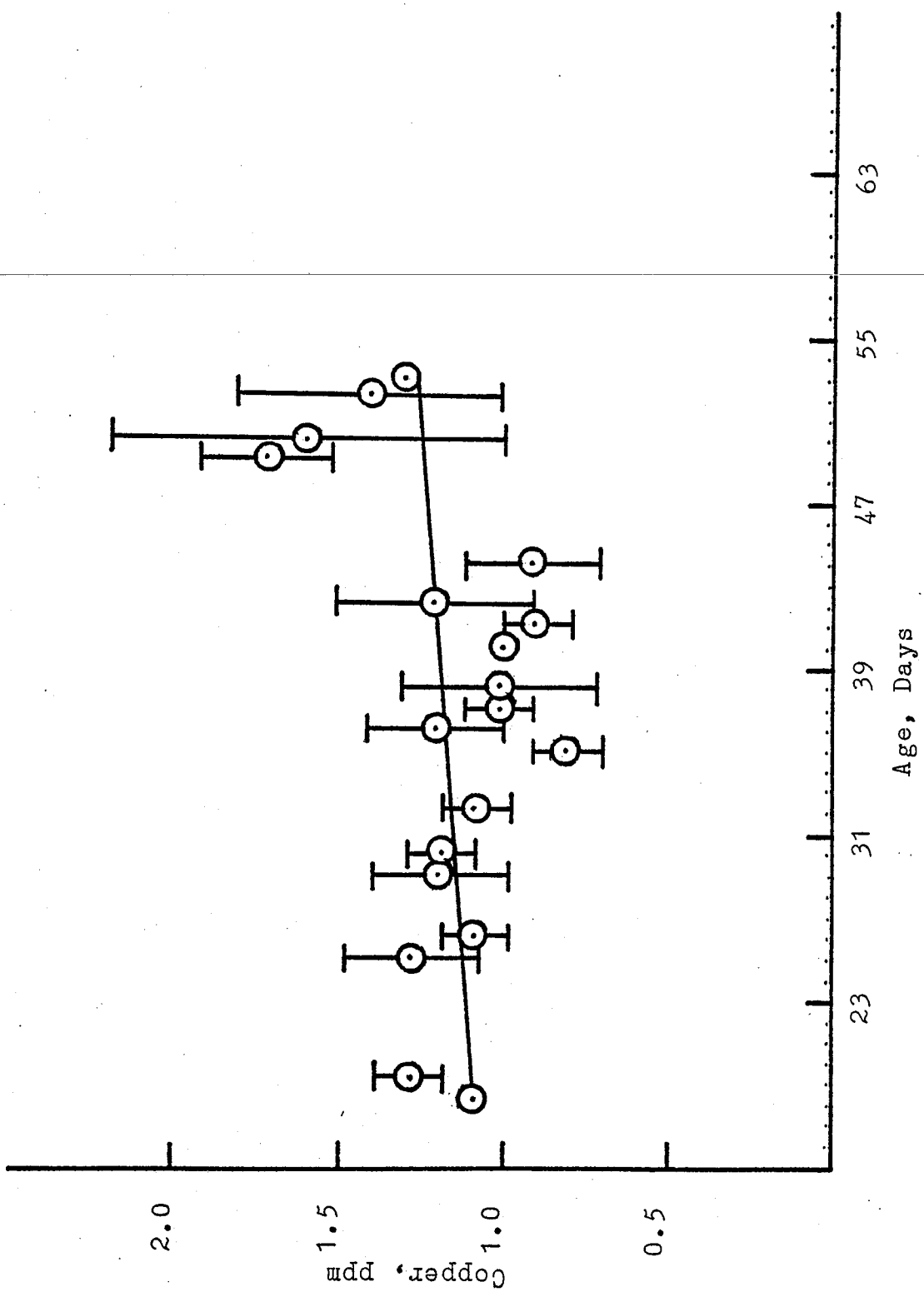

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.1.5

Male Plasma Copper Concentrations
versus Age for BALB/cJ Mice

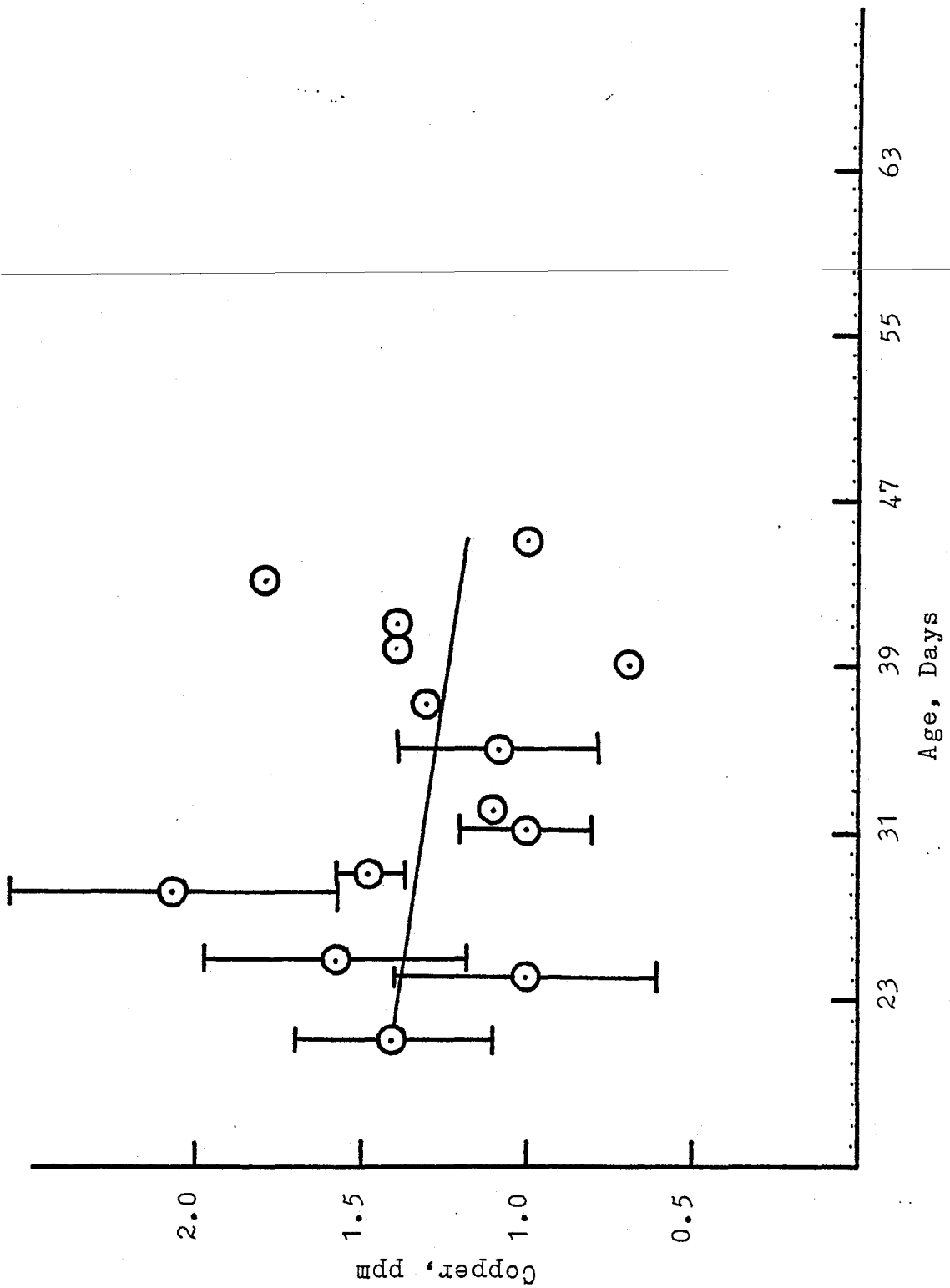

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.1.6

Male Plasma Copper Concentrations
versus Age for LP/J Mice

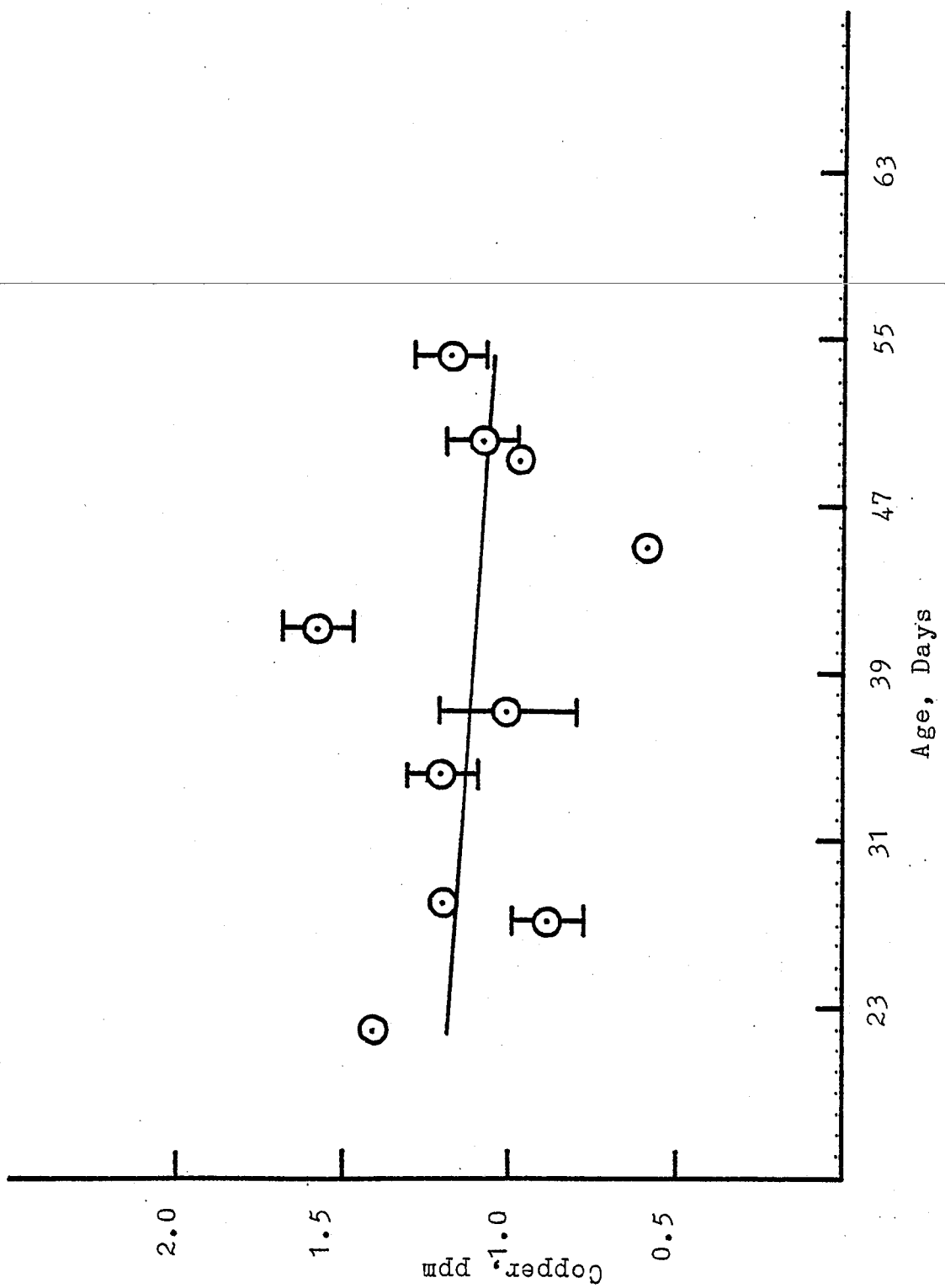

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.2.1

Female Plasma Copper Concentrations
versus Age for C57BL/6J Mice

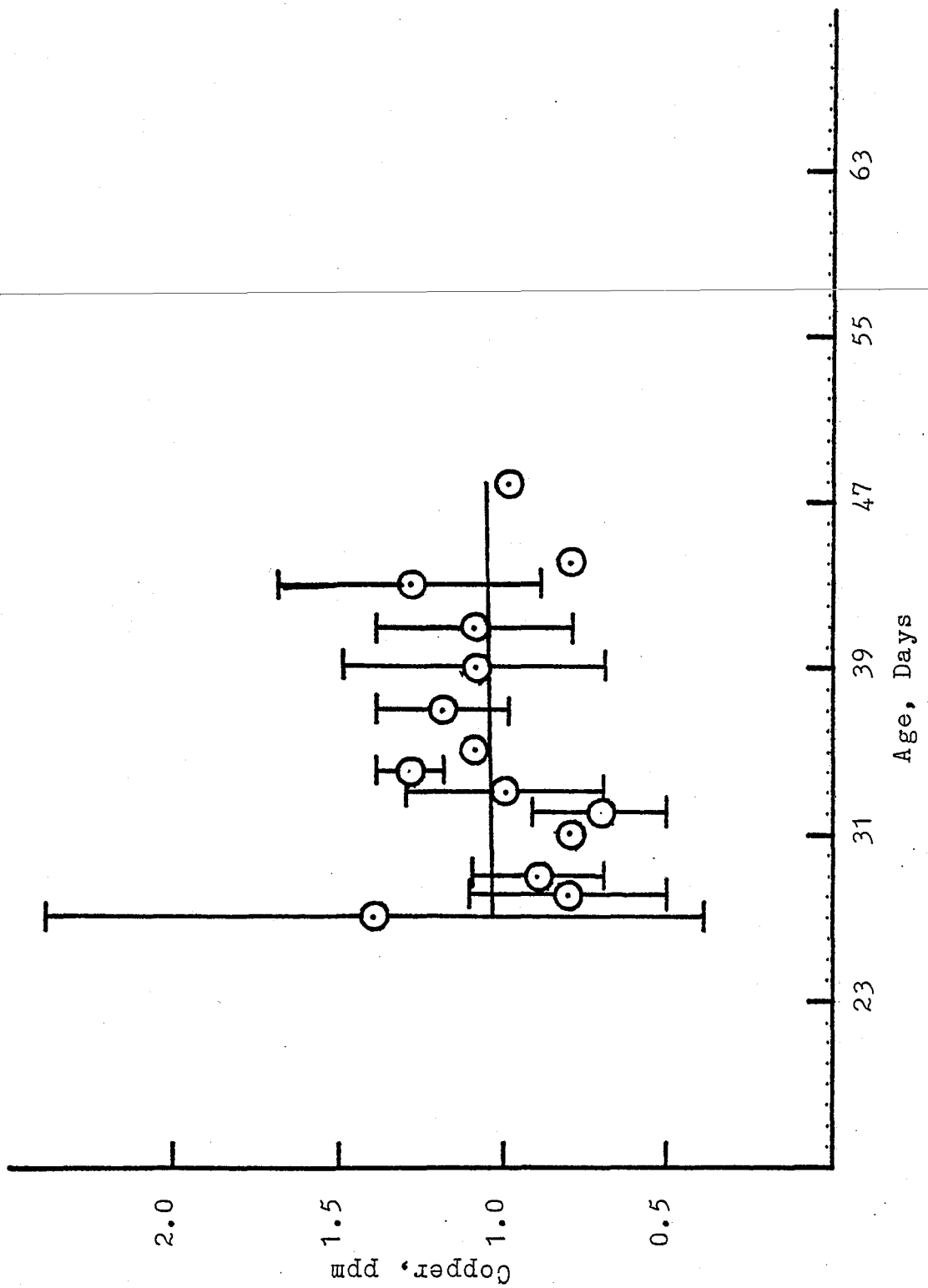

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.2.2

Female Plasma Copper Concentrations
versus Age for LG/J Mice

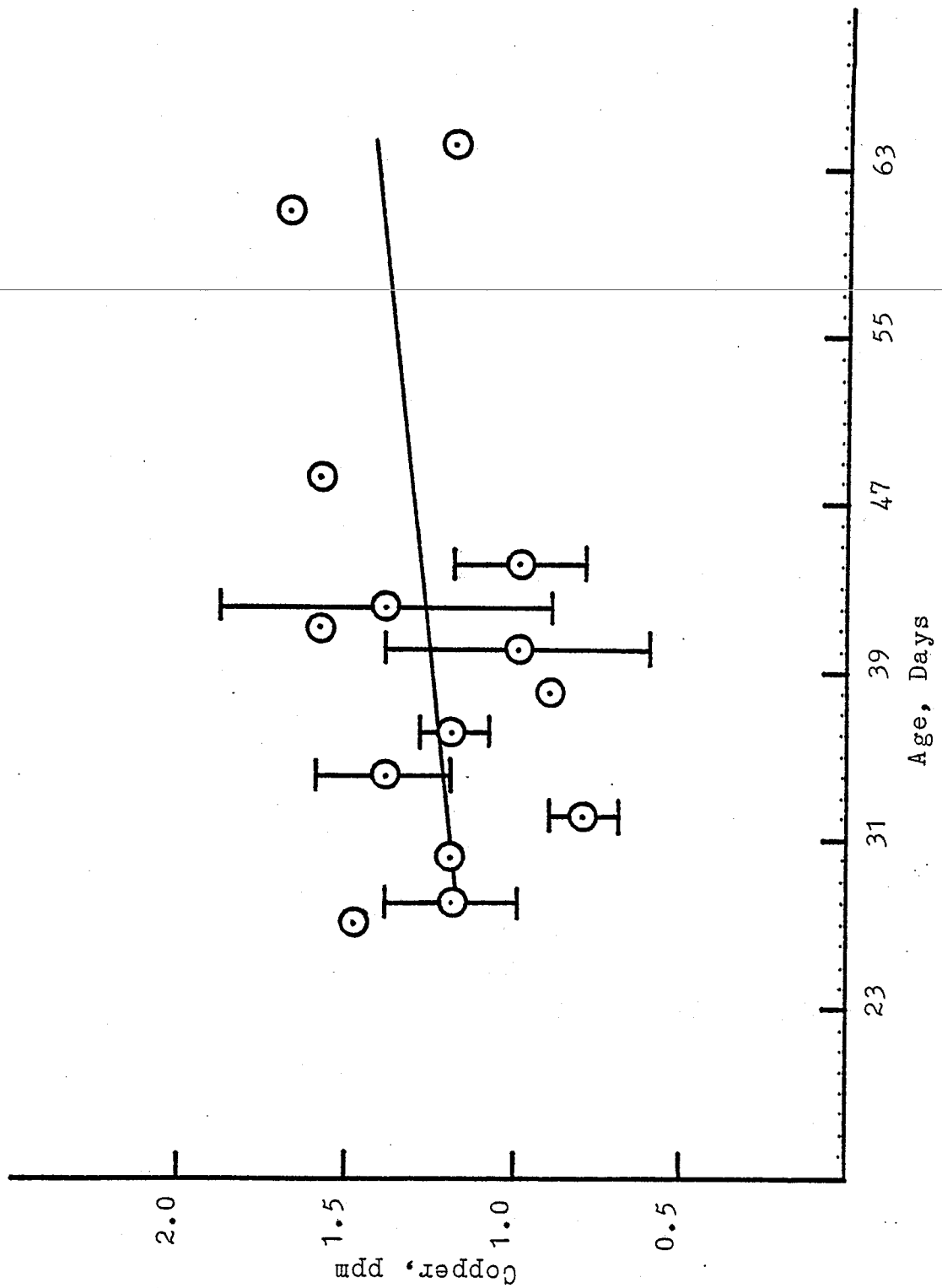

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.2.3

Female Plasma Copper Concentrations
versus Age for DBA/2J Mice

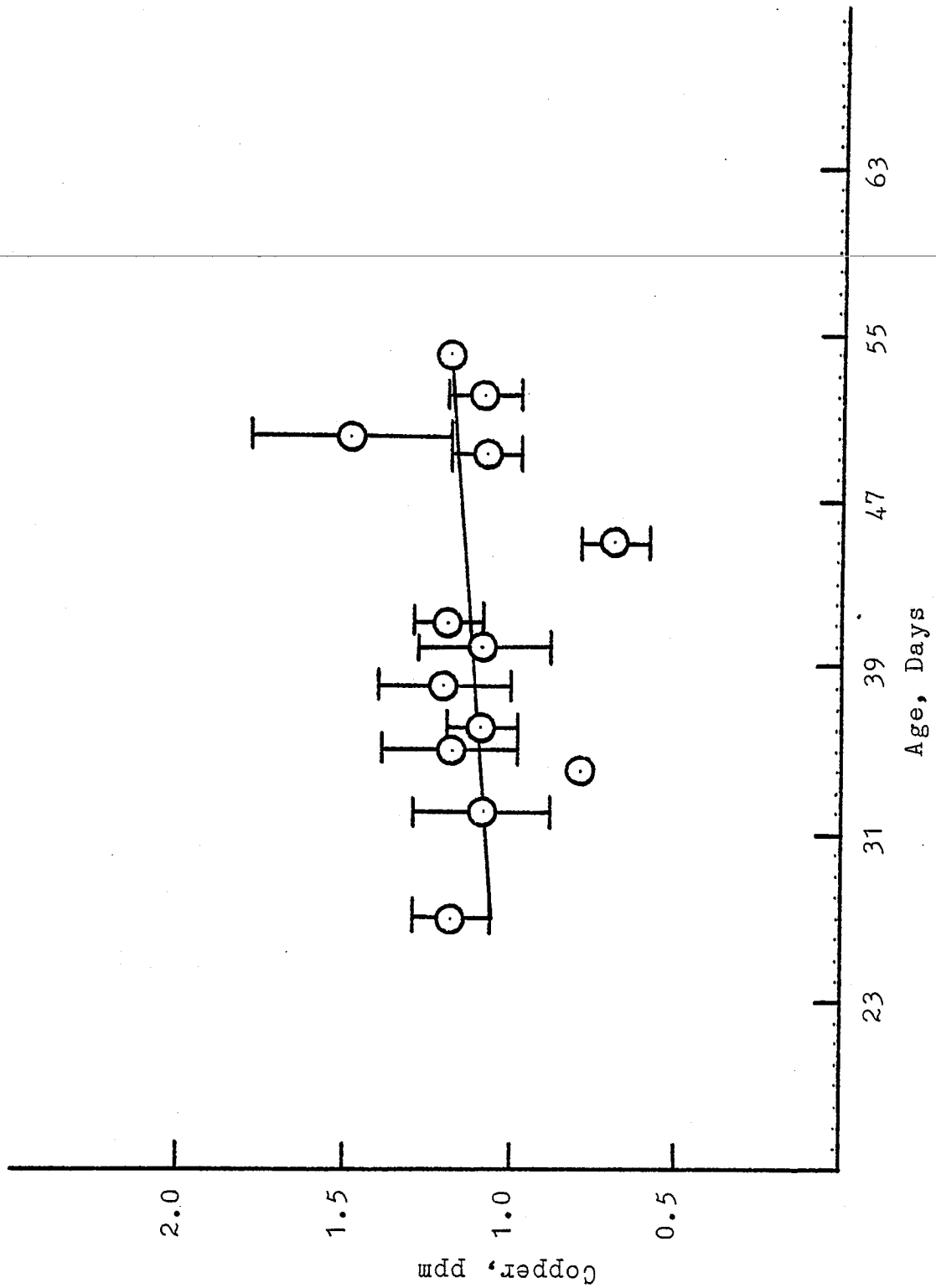

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.2.4

Female Plasma Copper Concentrations
versus Age for SJL/J Mice

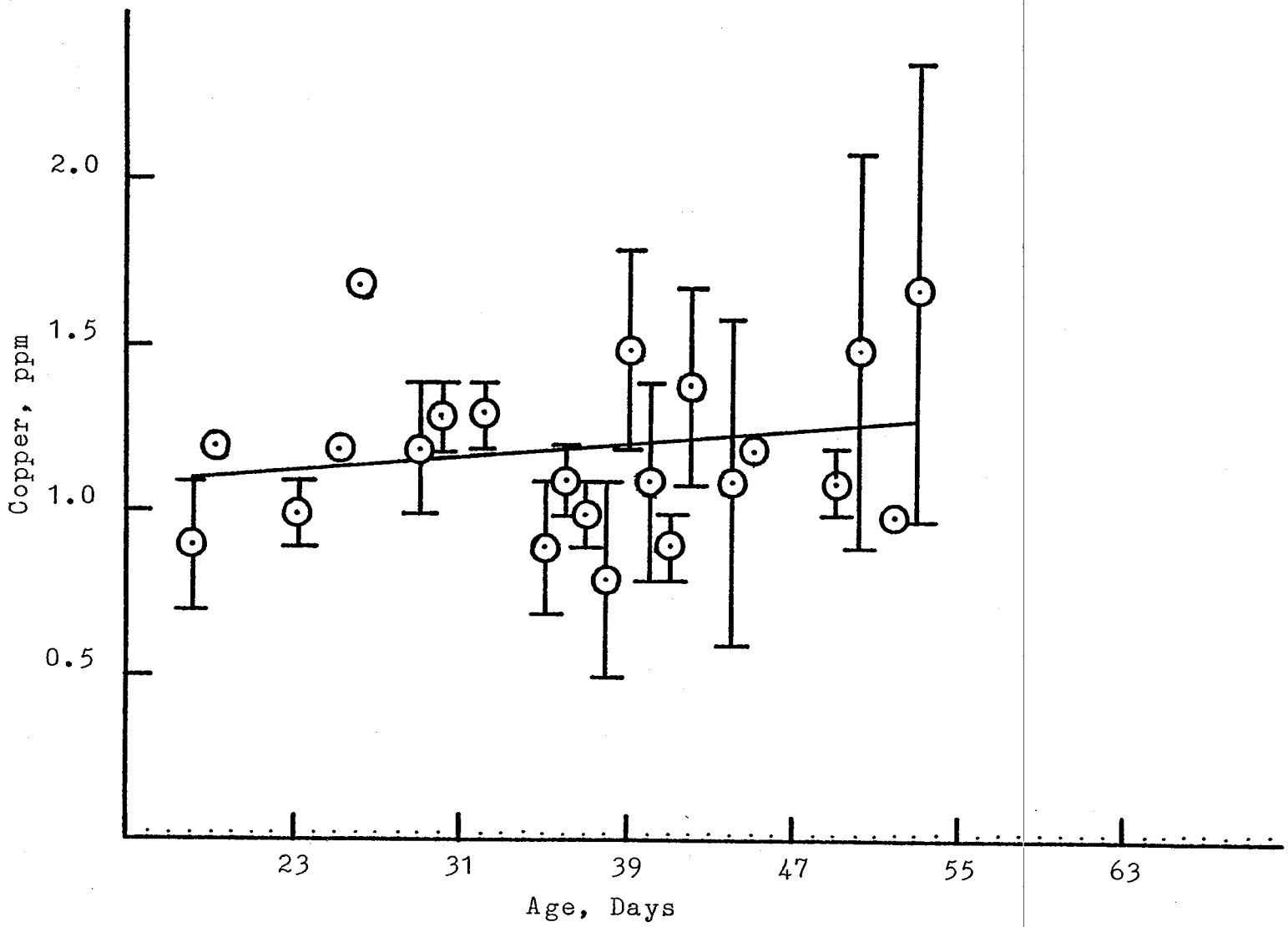

2.0
1.5
1.0
0.5
Copper, ppm
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.2.5

Female Plasma Copper Concentrations
versus Age for BALB/cJ Mice

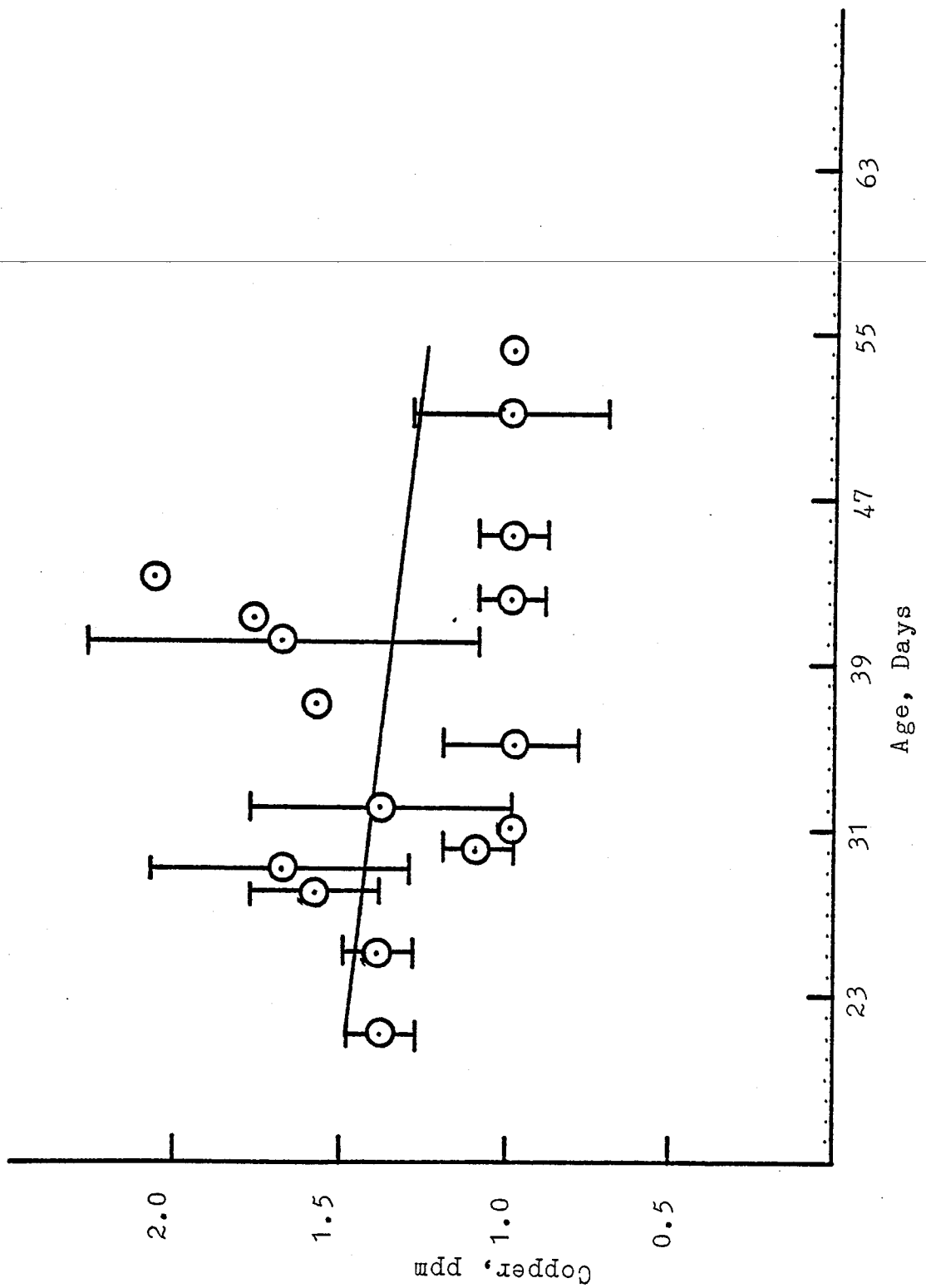
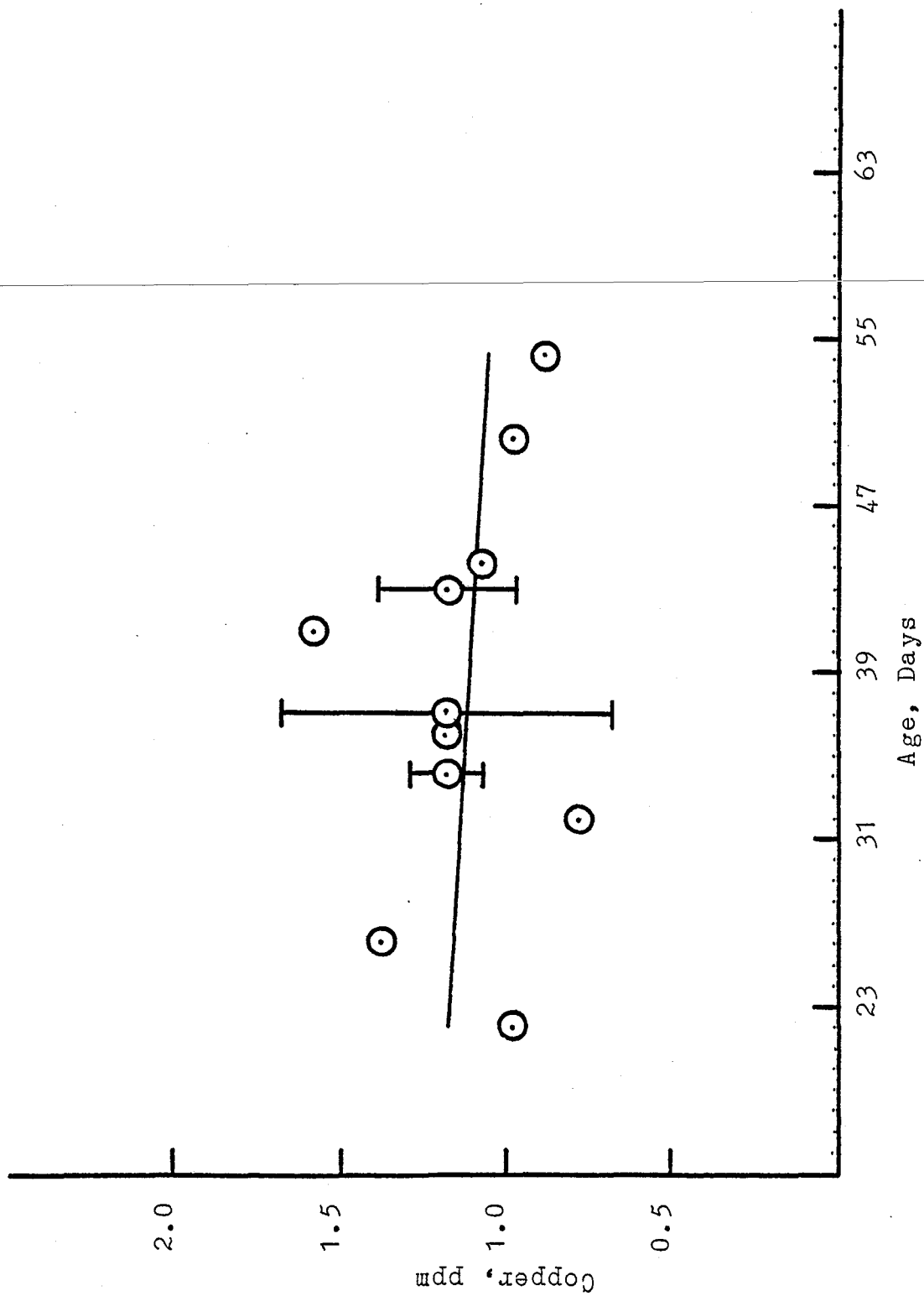

Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.3.2.6

Female Plasma Copper Concentrations
versus Age for LP/J Mice


Copper, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

A.1.4 Figures of Plasma Zinc Concentrations versus Age

FIGURE A.1.4.1.1

Male Plasma Zinc Concentrations
versus Age for C57BL/6J Mice

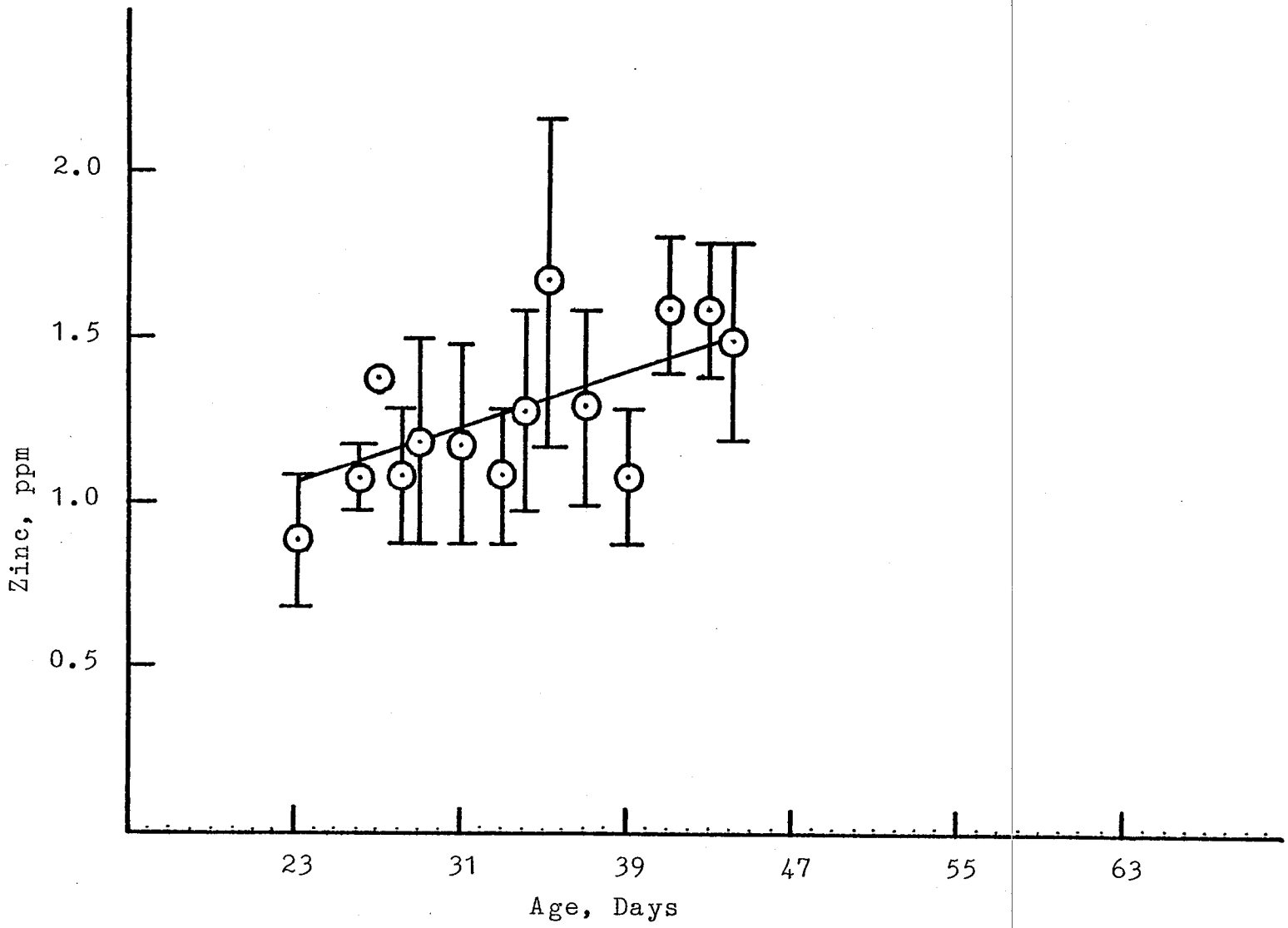

2.0
1.5
1.0
0.5
Zinc, ppm
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.1.2

Male Plasma Zinc Concentrations
versus Age for LG/J Mice

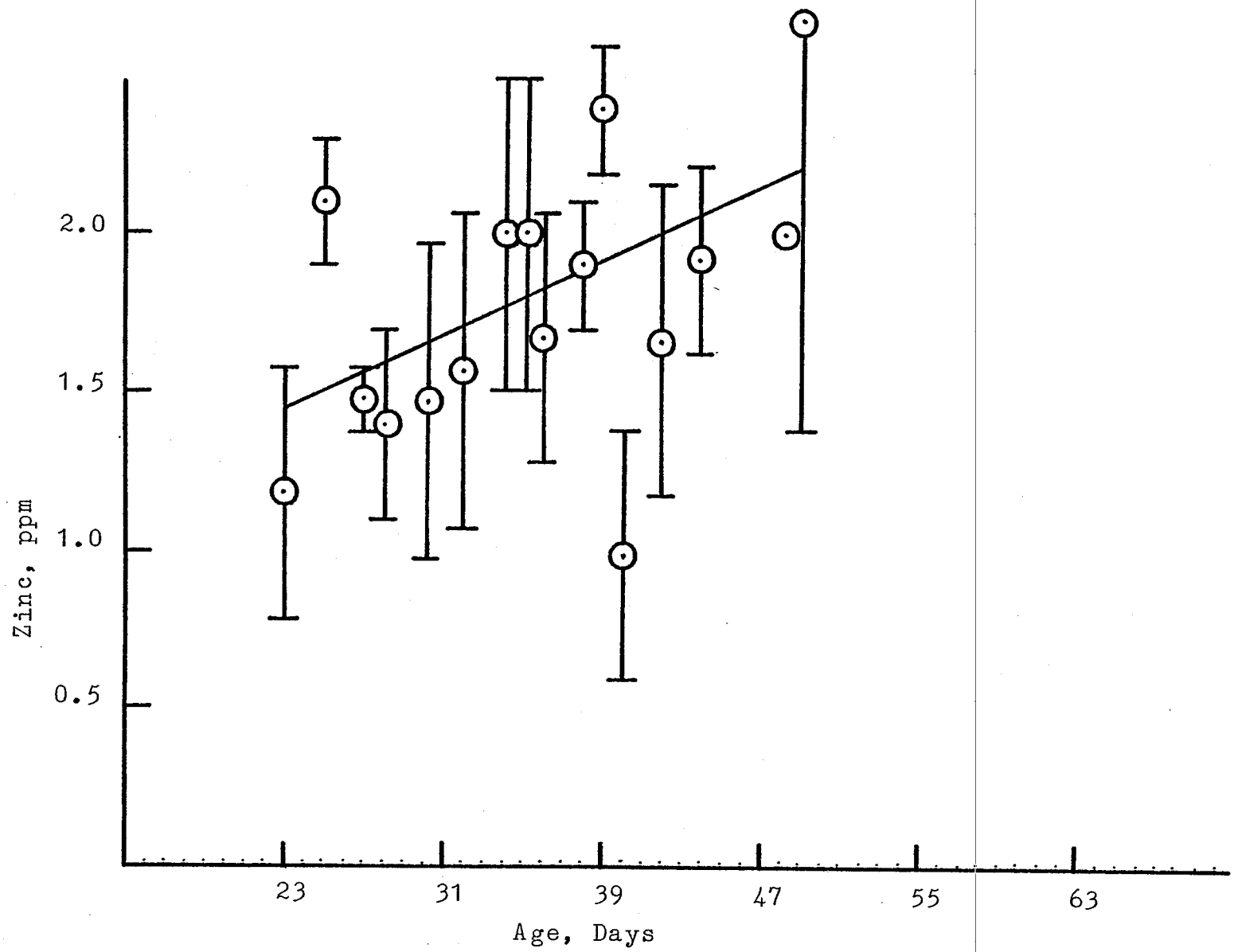

2.0
1.5
1.0
0.5
Zinc, ppm
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.1.3

Male Plasma Zinc Concentrations
versus Age for DBA/2J Mice

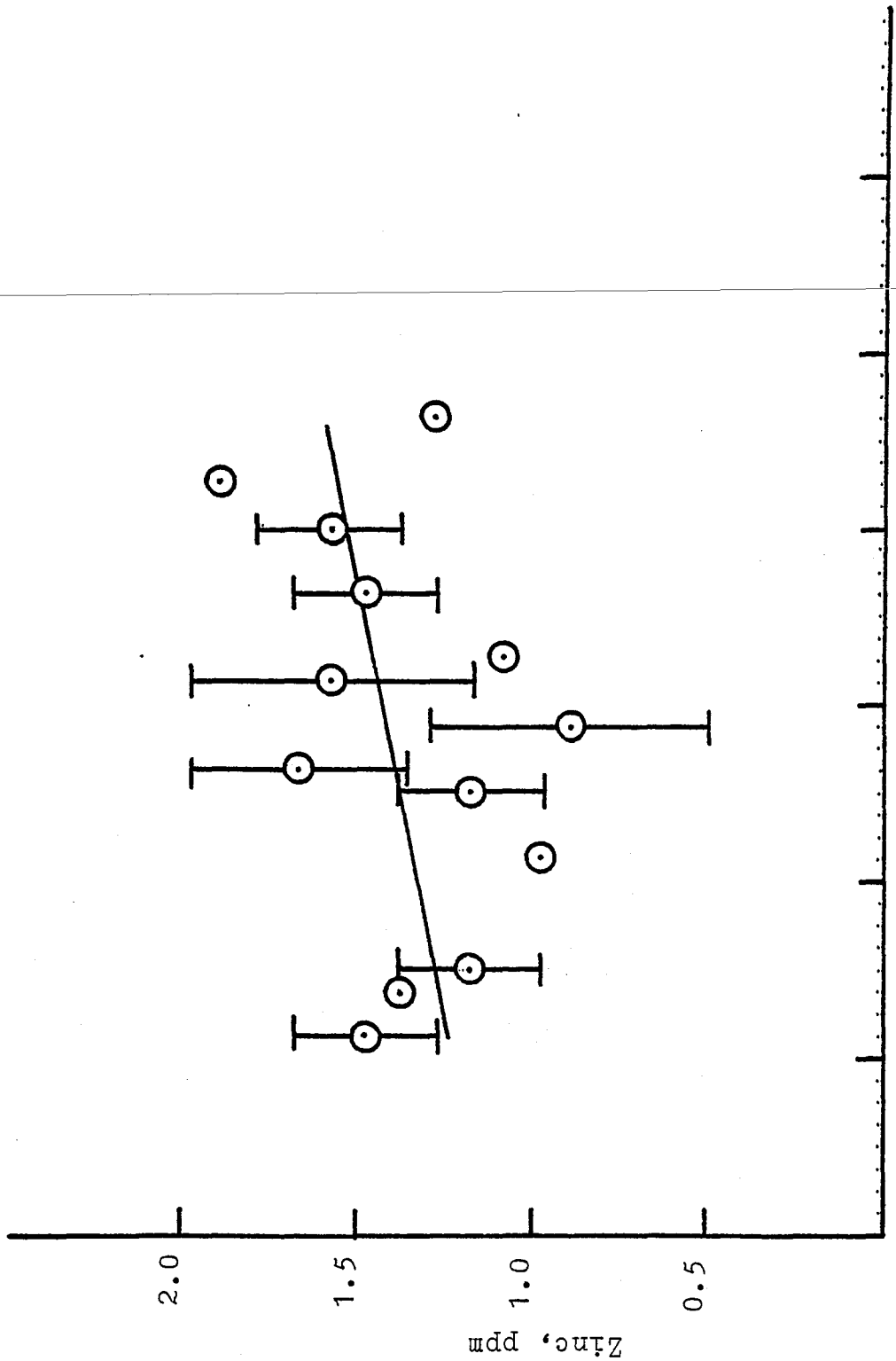

Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.1.4

Male Plasma Zinc Concentrations
versus Age for SJL/J Mice

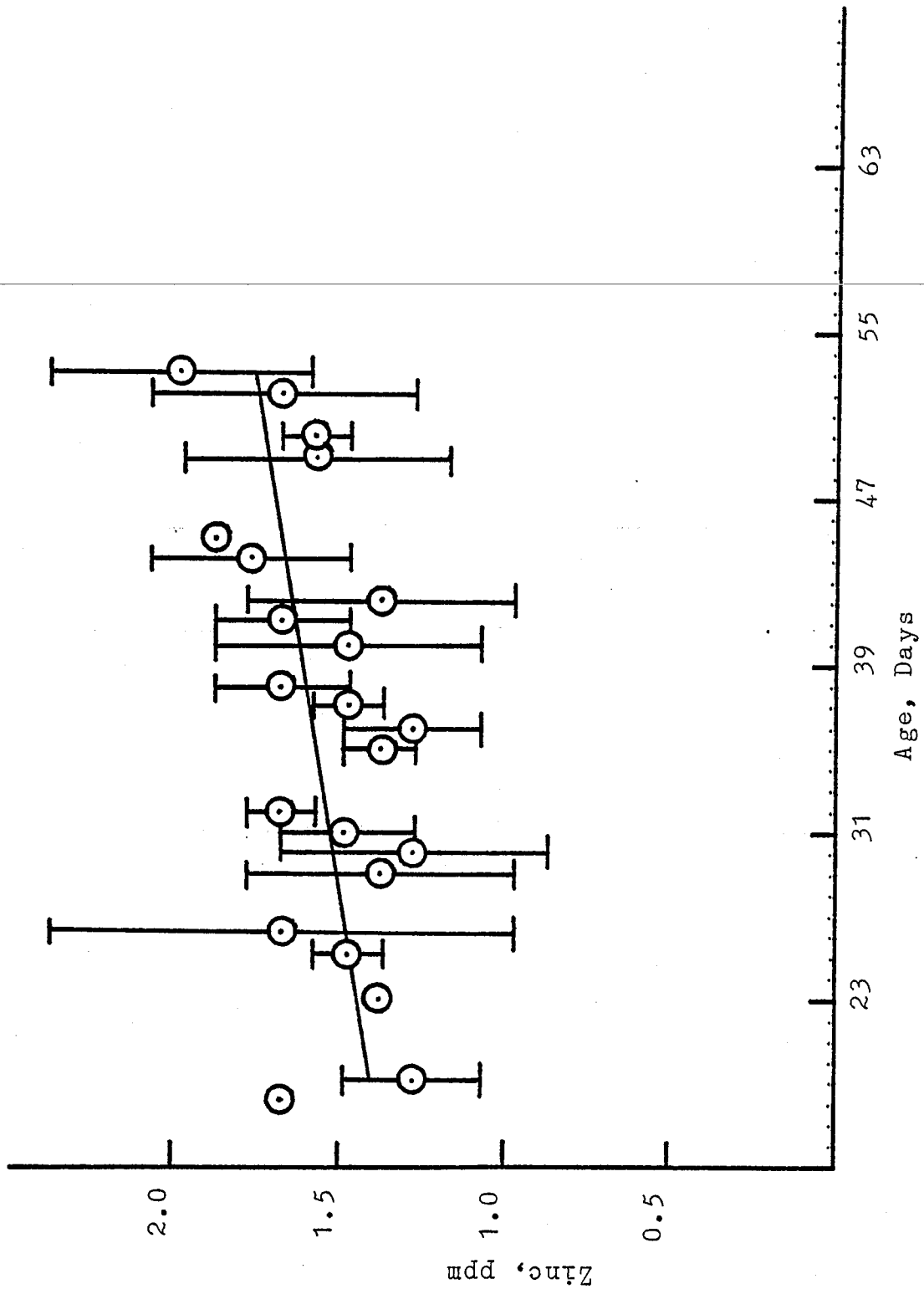

Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.1.5

Male Plasma Zinc Concentrations
versus Age for BALB/cJ Mice

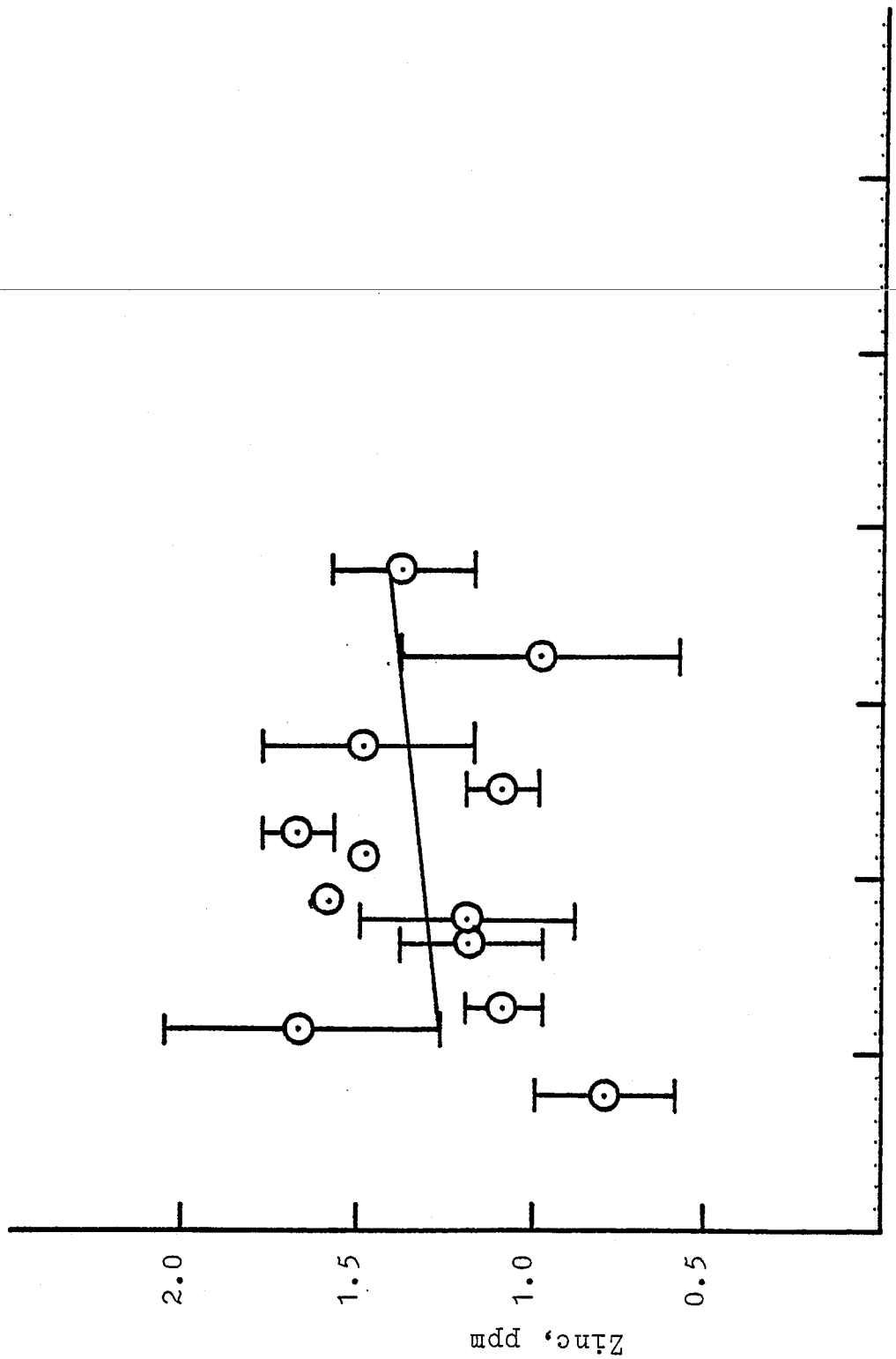

Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.1.6

Male Plasma Zinc Concentrations
versus Age for LP/J Mice

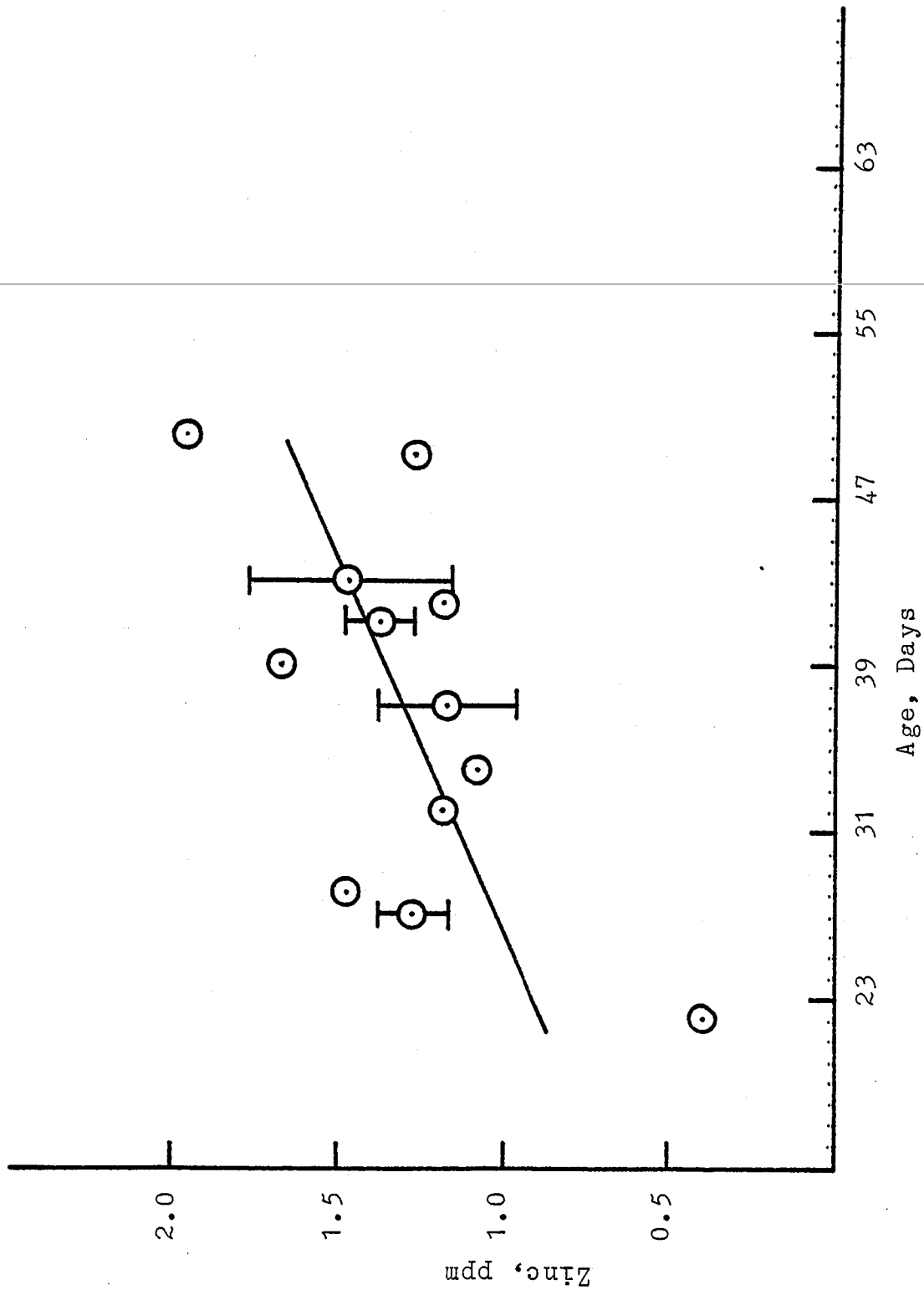

Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.2.1

Female Plasma Zinc Concentrations
versus Age for C57BL/6J Mice

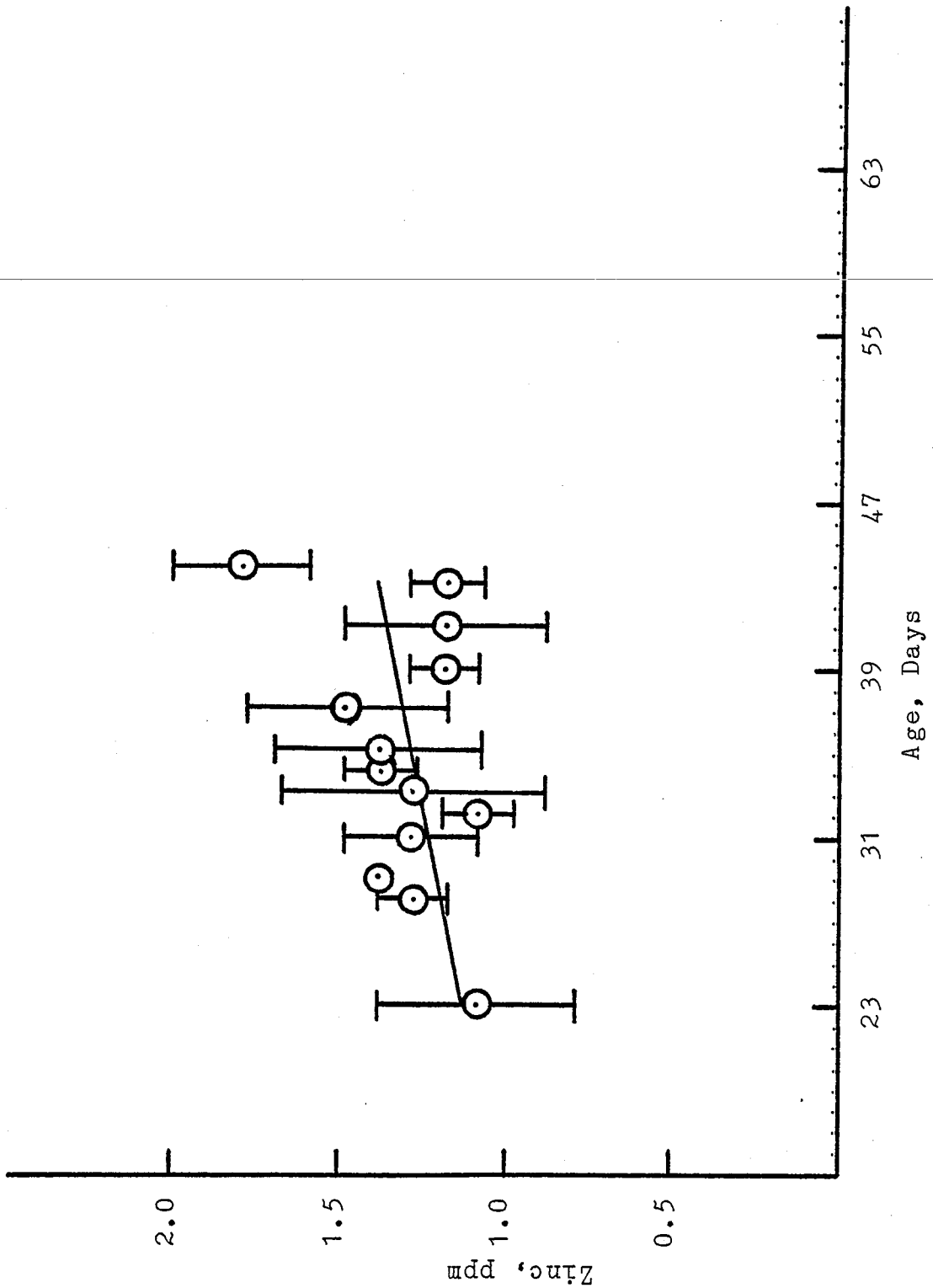

Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.2.2

Female Plasma Zinc Concentrations
versus Age for LG/J Mice

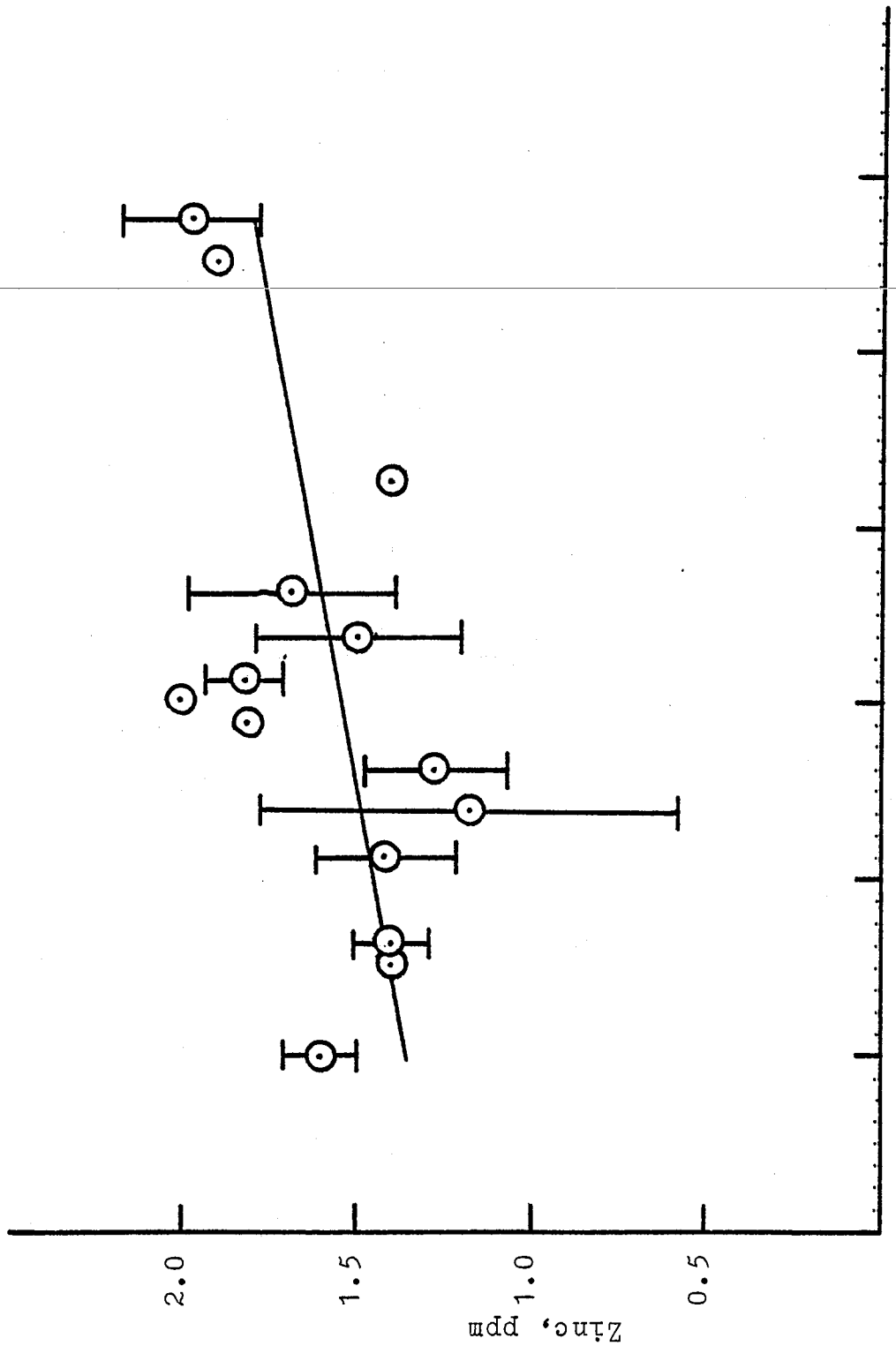

Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.2.3

Female Plasma Zinc Concentrations
versus Age for DBA/2J Mice

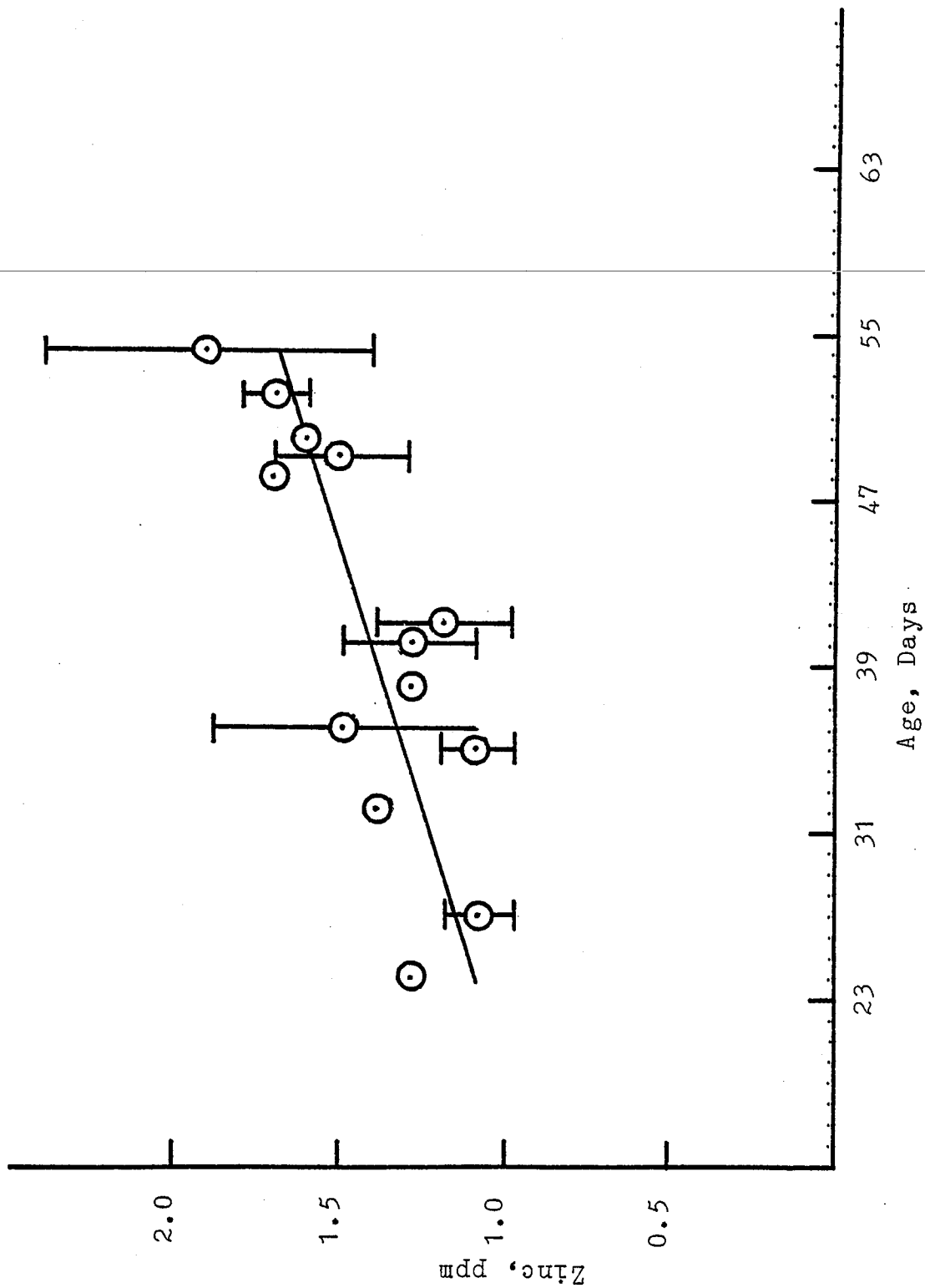

Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.2.4

Female Plasma Zinc Concentrations
versus Age for SJL/J Mice

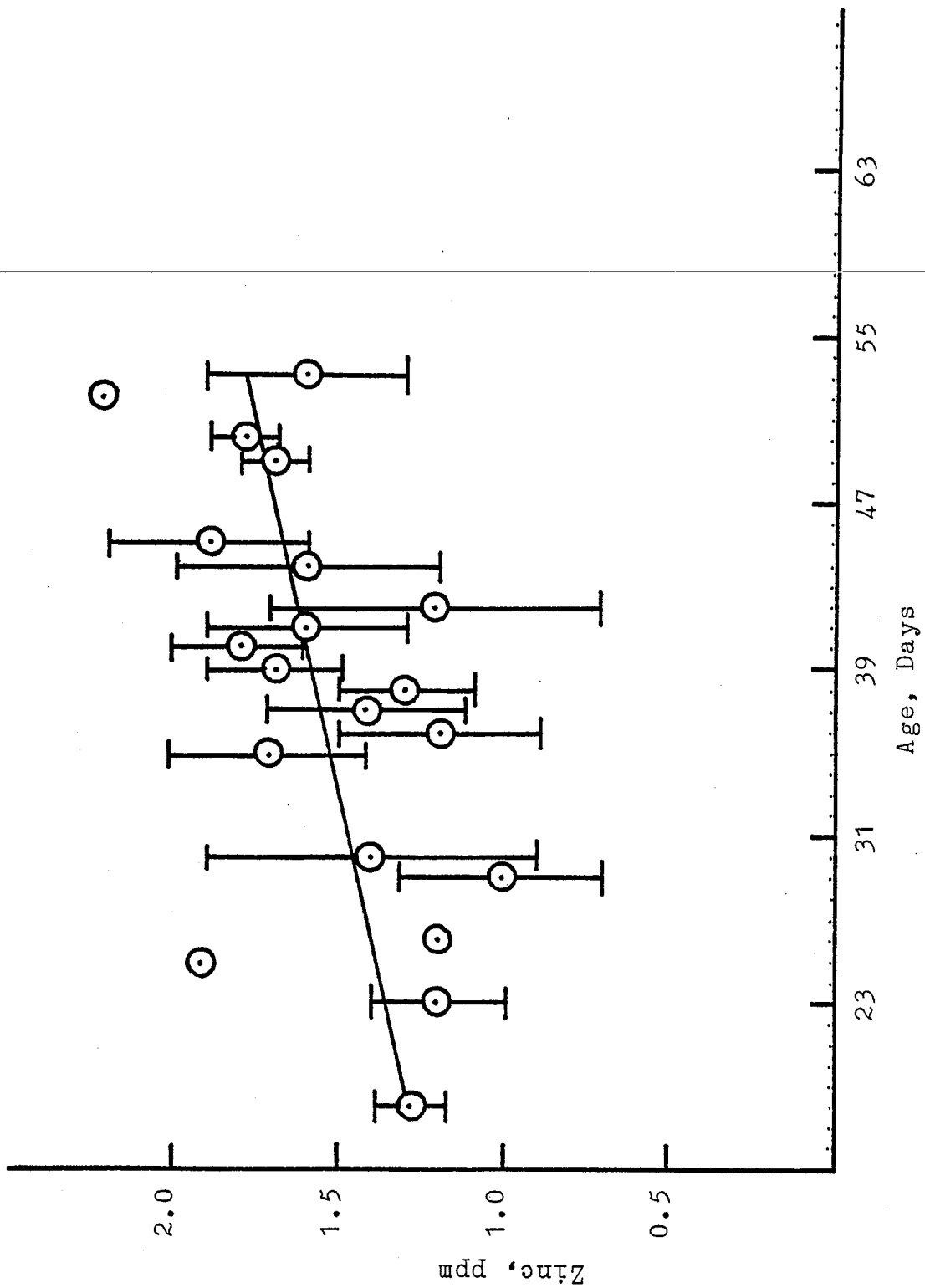

Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.2.5

Female Plasma Zinc Concentrations
versus Age for BALB/cJ Mice

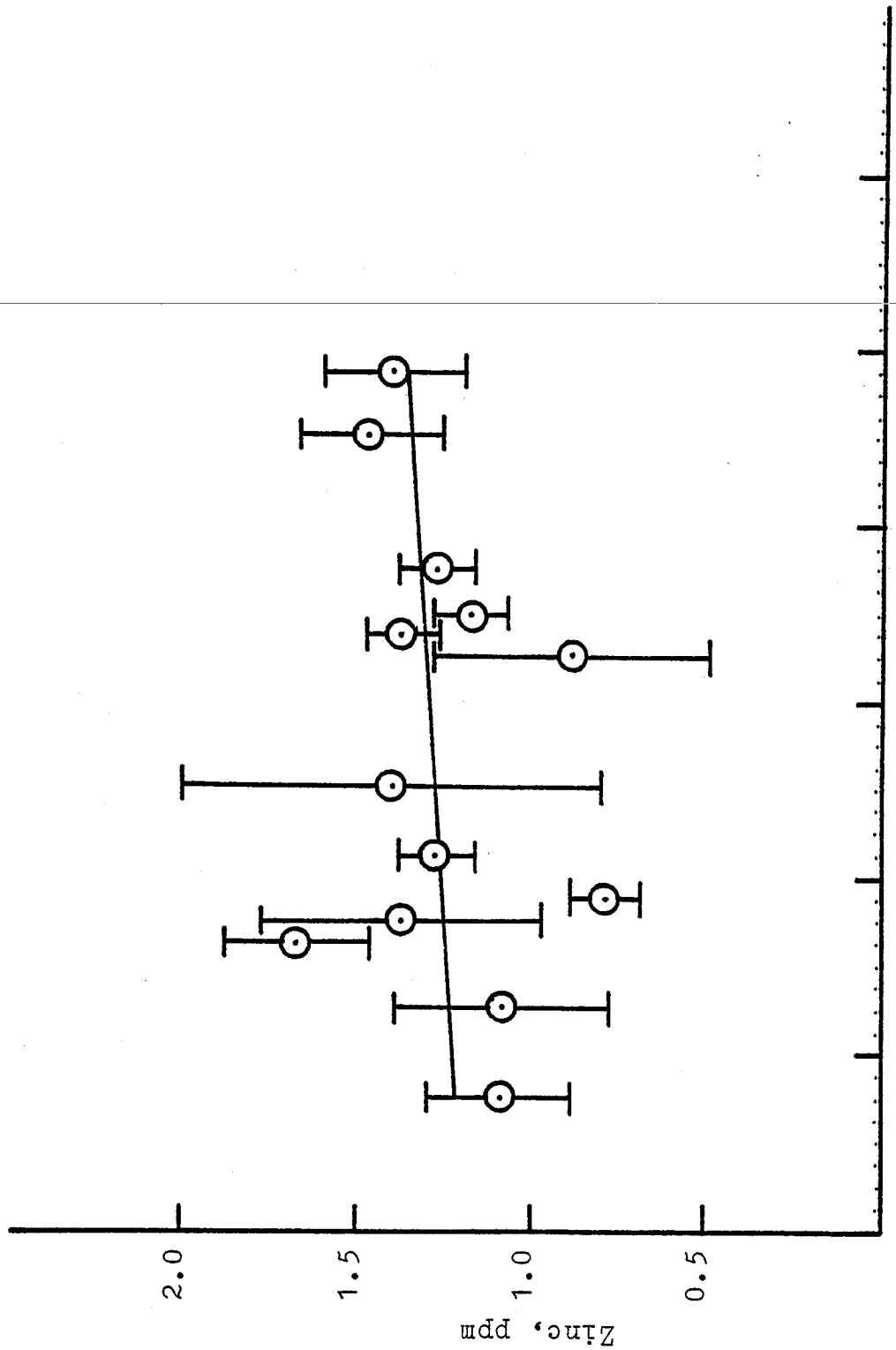
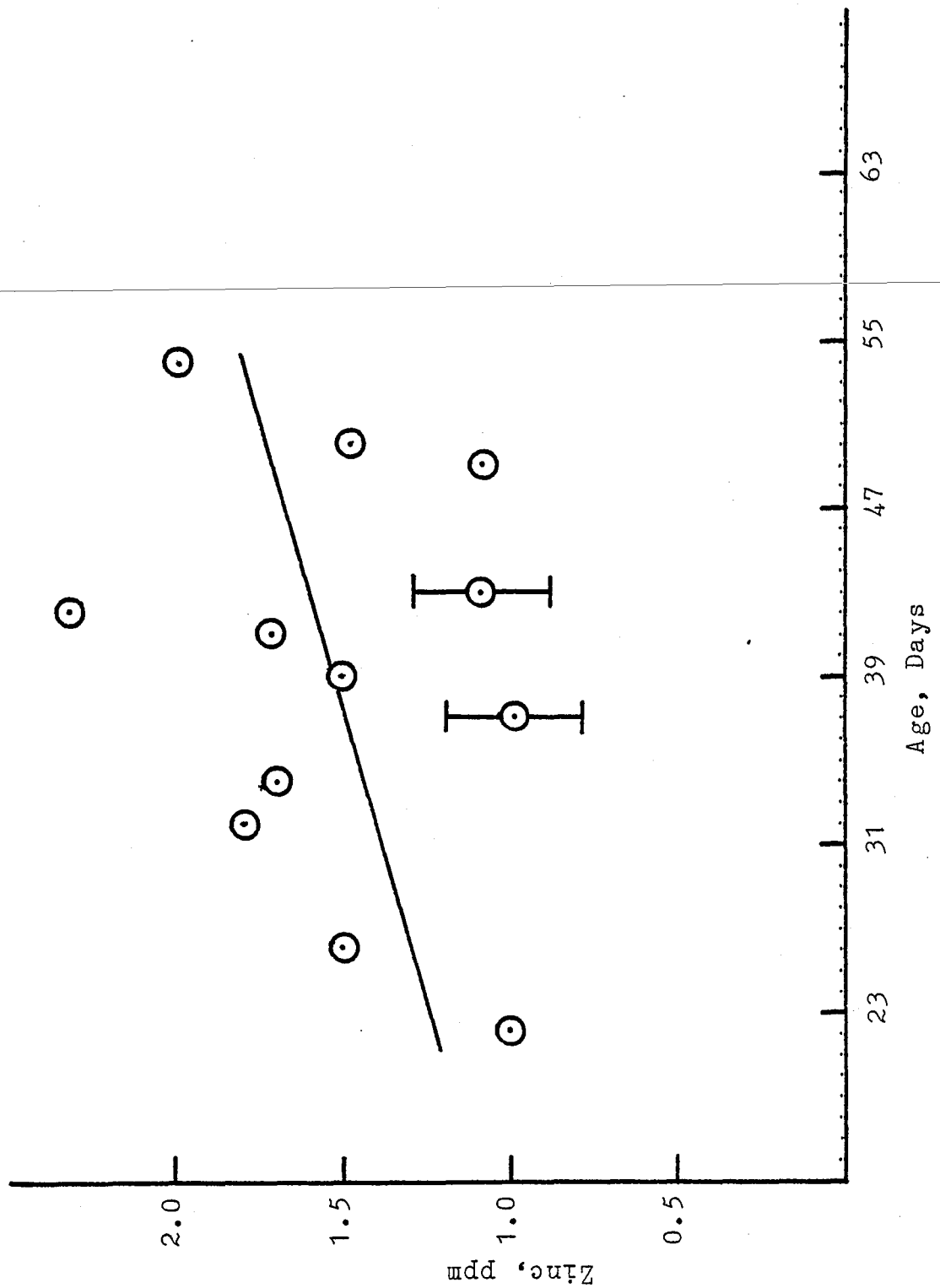

Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days

FIGURE A.1.4.2.6

Female Plasma Zinc Concentrations
versus Age for LP/J Mice


Zinc, ppm
2.0
1.5
1.0
0.5
23
31
39
47
55
63
Age, Days